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“Identification of metabolites in fermented foods by innovative analytical technologies”

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1. INTRODUCTION

1.1. Typicalness, quality and safety in EU food policy and innovative analytical approaches

The European Union's food policy is built around high food quality and safety standards: consumers should be offered a wide range of safe and high quality products coming from all EU Member States.

These standards of food quality and safety policy demand a comprehensive, dynamic and integrated approach. The food production chain is becoming increasingly complex, and therefore a successful food policy needs a traceability of feeding and food, and to facilitate such traceability, adequate procedures must be introduced.

The European Union activities are always directed to enforce the control safety and quality of food products. Furthermore the EU takes great care in the valorization and protection of the traditional agricultural and typical food-products and in designing rules to ensure that traditional foods are not forced off the market by means of its food standards, and by preserving the diversity of food market among different countries. When innovative analytical technologies are introduced, and new scientific knowdledge is acquired, new analytical ways have been considered open in order to characterize typical food products, to check or to improve their quality, their authenticity and to reveal possible food frauds.

Between the analytical techniques used for food quality and safety controls, the mass spectrometric techniques can give high analytical sensibility and allow wide analytical scope, overcoming difficulties related to complex matrices, as food matrices. These techniques present the intrinsic property to give structural information of analytes with

wide structural differences, and allow simultaneous quantification and identification of detected compounds and high potential for fast analysis time.

Advanced mass spectrometric techniques and global methodological approaches such as proteomics (study of structures and functions of proteins present in an organism; since proteins play a central role in an organism, proteomics is instrumental in the discovery of possible markers) and metabolomics (the systematic study of chemical fingerprints of specific cellular processes and of their small-molecule metabolite profile) may give important key information about the quality and typicalness of food products, also in the function of different making process.

Moving from these perspectives this PhD thesis dealt with the set up of innovative analytical methodologies, based on mass spectrometric techniques, aimed at characterizing and at improving the quality of typical food products by a "metabolomic" approach.

Mainly the research activities of this PhD thesis were directed at:

a) the determination of varietal odorous molecules (terpenes, norisoprenoids) and their precursors (terpene glycosides) present in grapes and in typical wines, specifically aimed at giving useful information about typicalness;

b) the characterization of proteins present in wines, with important enological functions related to their glycoprotein structure (parietal mannoproteins of *Saccharomyces cerevisiae*), in order to study structural variability and possible different release in wines depending on different strains of *S. cerevisiae* and on the technological process;

c) the aromatic characterization of Mozzarella di Bufala Campana cheeses aimed at obtaining useful information to improve the quality characteristics of this typical cheese.

1.2. Analytical characterization of typical food products

Generally food products are formed by matrices with a very complex chemical composition. The chemical characterization of some molecular compounds and metabolites present in foods, can give key information about their quality, safety and typicalness characteristics.

The analytical characterization of a typical food product can be carried out through the identification of its aromatic profile, which is one of the most important factors in determining food character and quality; some off-flavours can indicate critical points in its making process.

Furthermore, the characterization of a typical product can be carried out through the identification of non odorous metabolites (proteins, lipids, odorous precursors, etc.) which can be markers of its quality or typicalness.

1.2a Aroma molecules and aroma precursors in grapes and in wines

In grapes the composition of odorous molecules is often used for varietal differentiation, rational base of these studies is that the aroma compounds of grapes are constituted by several odorous molecules (alcohols, esters, acids, terpenes, ketones, aldheydes) and their concentrations can vary depending on variety [1].

It has emerged from numerous studies indicated that particularly terpenoid compounds, norisoprenoids, pyrazines, present in grapes, can form the axis for the varietal sensorial

expression of the wine bouquet which can be typical of its variety [2]. At present numerous monoterpene compounds and monoterpene diols in must and wine have been identified, and nearly 50 monoterpene compounds are known.

The dominating monoterpene alcohols, particularly from Muscat varieties, seem to be linalool, geraniol, nerol, citronellol, α-terpineol, while in the case of koshu, an indigenous Japanese variety, terpinene-4-ol was identified as the dominating monoterpene compound [2].

Terpenes seem to have an ecological significance in plants [3], for instance, in the interaction between plants and micro-organisms, they can act as repellents against aphids [4]; they can be released as semiochemicals after herbivore attacks [5-7], and as such they may attract predators of the herbivores [8], and obviously they can be responsible for attracting pollinating moths [9].

Monoterpenes are secondary plant constituents and they seem to be formed by biosynthesis, while C13 norisoprenoids seem to result from biodegradation of diterpenes and carotenoids [10].

The biochemical pathways leading to the formation of monoterpenes and their derivatives is not completely known, but the relation between terpene composition and grape variety is connected to the existence of reactions catalysed by enzymes.

Genes encoding enzymes catalysing the biosynthesis of the monoterpenes S-linalool [11,12]; R-linalool [13]; (±)-(4S) limonene [14,15]; myrcene, (±)-(1S,5S)-pinene; myrcene and (E)- bocimene [16]; (+)-bornyl diphosphate, 1,8-cineole, (+)-sabinene [17]; (±)- β-phellandrene, (±)-camphene, terpinolene and one making (±)- limonene and (±)- a-pinene [8] have been isolated in the past few years from a number of plant species.

In order to give a classification of monoterpenes, three types of categories of monoterpenes can be considered in grapes:

1) free aroma compounds, commonly dominated by linalool, geraniol, and nerol, together with the pyran and furan forms of the linalool oxides, citronellol, α-terpineol, ho-trienol, nerol oxide, myrcenol, the ocimenols plus several other oxides, aldehydes and hydrocarbons. Several monoterpene ethyl ethers and acetate esters have also been found among the free aroma compounds;

2) the polyhydroxylated forms of the monoterpenes, or free odourless polyols. Although these compounds make no direct contribution to the aroma, some of them seem to be reactive and to break down to give pleasant and potent volatiles.

3) glycosidically conjugated forms of the monoterpenes which make no direct contribution to the aroma of grape. Glycosides are, in most cases, more abundant than the unglycosilated forms of individual monoterpenes [2].

Glycoside precursors are numerous and abundant, in flavourant grapes they are evaluated between 6.5 and 28 mg/ l of juice.

The sugar moieties observed were rutinosides (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside), 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides, 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides or β-D-glucopyranosides. The aglycon part is often formed with terpenols, linalool oxides, terpene diols and triols.

However, other flavour precursors can occur such as alcohols, e.g. hexanol, phenylethanol, benzyl alcohol, norisoprenoids, phenolic acids and probably some volatile phenols.

Some studies indicated that abundant glycosides in some grapes were apiosylglycosides (up to 50% according to grape variety), followed by rutinosides (6–13%) and glucosides (4–9%). A more accurate analysis seems to indicate that all glycosides are not present in

all cultivars and that their proportions also differ according to grapes. Some glycoside flavour potentiality of grapes remains quite stable during winemaking and in young wines as well [2].

A number of surveys have been made of monoterpene concentration in different grape varieties. A general classification of grape varieties is possible allowing division into (1) intensely flavoured muscats, in which total free monoterpene concentrations can be as high as 6 mg/l; (2) non-muscat but aromatic varieties with total monoterpene concentration of 1–4 mg/ l; and (3) more neutral varieties with lower concentrations.

It is just from cultivars of group (3) that a large volume of the world's wine comes.

Terpene glycosides can be hydrolysed by acids or enzymes. Aging and storage provide wine with the time for the slow transformation of the free and bound monoterpenes. It is generally believed that hydrolysis of glycosides and polyols into aromatic monoterpenes is faster than free monoterpene isomerization and rearrangement [18]. Experiments on both whole juice and monoterpene glycosides isolated from juice, have demonstrated that significantly different patterns of volatile monoterpenes are produced when each is hydrolysed at different pH values in some conditions of temperature: acidic hydrolysis of terpene glycosides can provoke a molecular rearrangement of the monoterpenols which are transformed in other compounds.

To enrich wine flavour by means of release of free aromatic compounds from natural glycoside precursors, particular pathways are required. Enzymic hydrolysis is the most interesting because it produces a more ‘‘natural’’ flavour in wine, it is carried out with various enzymes which act sequentially according to two steps: at first, α-L-rhamnosidase, α-L-arabinosidase or β-D-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose or apiose and the corresponding β-D-glucosides are released; subsequently liberation of monoterpenol takes place after action of a β-D-

glucosidase. Some studies reported that the grapes have an enzyme with β-glucosidase activity but only low α-rhamnosidase, α-arabinosidase or β-apiosidase activities have been detected. Enzymic hydrolysis of glycoside extracts from Muscat, Riesling, Semillon, Chardonnay, Sauvignon and Sirah varieties have provoked the liberation not only of terpenes, but also C13-norisoprenoids, such as 3-oxo-α-ionol and 3-hydroxy-β-damascenone. These compounds are capable of awarding certain typicity to the wine flavour because they have lower threshold values than terpenes and they contribute to aromatic features.

Several exogenous enzymes, mainly with a fungal origin, have been developed to liberate terpenes in wines.

The most suitable enzymic preparations to be used during winemaking process are those which possess all β-D-glucopyranosidase, α-L-arabinofuranosidase, α-L-rhamnopyranosidase and β-D-apiofuranosidase activities. These enzymes are present only in low quantities in the majority of commercial fungal enzymic preparations, mainly regarding β-D-apiosidase activity. Some studies indicate that the concentrations of some free volatile compound terpenols, norisoprenoids and volatile phenols in enzyme treated wines highly increase, not only in aromatic varieties, but also in neutral ones, from 265 to 2000% .

The ability of purified enzymes, synthetic substrates and suitable analytical methods are needed for the rigourous progress in the field of enzymic hydrolysis of terpenyl glycosides. Some enzymes have been isolated from fungal enzymic preparations, vegetal extracts or synthetic culture media inoculated with fungal cultures (mainly *Aspergillus niger*), and have been purified by means of different chromatographic

techniques (gel filtration, ion-exchange chromatography, affinity chromatography, chromatofocusing).

Some pectinolytic preparations (obtained generally from GRAS microrganisms Aspergillus spp.) are largerly used in winemaking process to improve juice clarification, juice yeld, and color extraction [19]. In addition to their main activities, these preparations possess other enzyme "side activities" including glycosidases, which are remarkably stable in wine pH in contrast with those from plant and yeast. It is important to notice that pectinolytic enzyme preparations differ largerly in glycosidase activities involved in aroma release since they are formulated as functions of their pectinase activities.

The analysis of odorous molecules of grapes allow the monitoring of grape maturation: traditionally grape maturity corresponds to an optimal sugar/acid ratio, however the production of quality white wines requires grapes whose aromatic substances are at a maximal concentration [1].

Volatile compounds of grapes are generally present in trace amounts and require a previous step of isolation and concentration for the subsequent gas chromatography analysis. Different methods have been used in order to isolate volatile compounds from must such as simultaneous distillation-extraction or solid-phase extraction. Dynamic headspace technique has been used to analyse the volatile composition of grapes in order to discriminate different varieties.

Solid-phase microextraction (SPME) is a fast, simple and solvent-free technique that with different types of adsorbents with a wide range of polarity, makes it possible to isolate trace compounds of different substrates. Traditional fibres (PDMS and PA) have been used to determine terpenes and fermentation compounds in wines, fruits and juices, however these fibres present a poor sensitivity for polar compounds.

Mixed coating fibres containing divinylbenzene (DVD), PDMS and carboxen (CAR) or polyethylene glycol (CW), increase the tramping ability of the fibre due to the synergic effect of adsorption and distribution within the stationary phase, producing higher sensitivity than PDMS and PA fibres. Previous studies have shown that some SPE polymeric sorbents also provide highest solid–liquid distribution coefficients.

In wine the aroma is one of the most important factors in determining its character and quality.

More than 99% of a wine flavour extract is composed of fusel alcohols, fatty acids and fermentation esters, whereas the remaining 1% of the extract (which contributes significantly to the bouquet of a wine) is composed of hundreds of compounds which are present at concentrations nearly 10^{6}-10^{8} times lower than the fusel alcohols. Many of these compounds are present at concentrations which are higher than their odorous threshold [20].

The varietal characteristics of aroma bouquet of a wine derive mainly from the presence of varietal compounds as terpenes, norisoprenoids and pyrazines.

Some aroma compounds can be odorous markers of specific technological process. Aromatic molecules released by barriques can be for example: 2-methoxy-phenol (guaiacol), aromatic descriptor spices), 4-ethyl phenol (ink), 4-metyl guaiacol (medicinal), eugenol (cloves), vanilline (vanilla), acetovanillone (vanilla), butyrolactone (butter), 5-ethyl furfurale, ethyl hexyl disulphide.

The qualitative and quantitative analysis of the volatile compounds of wines present several analytical difficulties due to 1) the complex chemical composition of the volatile fraction and 2) the fact that individual volatile compounds can be present in a wide range of concentration (from 1 ng/l to several g/l).

Analytically the odor-active molecules of wine can be classified into the following categories: 1) Category 1: compounds easily accessible from the analytical point of view. This group comprises all the compounds present at a relatively high concentration (*C* 0.1 mg/ l) which can be determined after a single isolation step and a GC–flame ionization detection (FID) analysis. Acetaldehyde, higher alcohols and some of their acetates, fatty acids and their ethyl esters are typical examples [21].

2) Category 2: Compounds of intermediate analytical accessibility. The analysis of these compounds is possible after a powerful isolation-preconcentration step and further GC–MS. Generally speaking, these are compounds with a reasonably good chromatographic behavior present at concentrations between 0.1 μg/ l and 0.1 mg/ l. Compounds in this group are volatile phenols, some lactones, vanillin derivatives, some minor esters, and some norisoprenoids, such as β-damascenone and β- ionone [21].

3) Category 3: Compounds of very difficult analytical accessibility. This is a heterogeneous group formed by compounds whose analysis is very difficult for different reasons, such as bad chromatographic behavior and poor chemical stability [22], extremely low concentrations [23], or very poor analytical properties. Volatile sulfur compounds [24,25], aldehydes [26], alkyl methoxypyrazines [27,28], furaneol and sotolon [29,30], and some aromatic thiols [31] are well-known examples of this category. In general, the analysis of these compounds requires the development of specific methods of isolation, or detection, or the use of chemical derivatives.

In literature a few examples of analytical methods for studying the composition of terpene glycosides are present and the examples reported concern aromatic grapes mainly [32-35]. Some of these studies were effected by GC/MS analysis of TMS-derivatives of terpene glycosides and some others were carried out through the analysis

of terpenes obtained through hydrolysis of terpene glycosides by means of purified enzymes or commercial enzymatic preparations.

A few studies were carried out on terpenes and glycoside terpenes of non aromatic grapes; furthermore there aren't any analytical studies for glycosides of grapes through LC/ESI/MS or MALDI/TOF/MS techniques (which would allow to identify glycosides just like they are, without derivatization reactions which are necessary for GC/MS analysis).

1.2b Mannoproteins from *Saccharomyces cerevisiae*: proteins with important enological functions present in wines

Mannoproteins, which make up 35 –40% of the cell wall of Saccharomyces cerevisiae, are glycoproteins, often highly glycosylated, located in the outermost layer of the yeast cell wall, where they are connected to a matrix of amorphous β-1,3 glucan by means of covalent bonds [36,37]. The degree of mannoprotein glycosylation is variable; in some cases, mannoproteins can contain over 90% sugars, mainly mannose [36].

Mannoproteins give the yeast cell wall its active properties and play an important role in controlling the wall's porosity [38-40], thereby regulating leakage of proteins from the periplasmic space, and entrance of macromolecules from the environment.

At different pH values, the electrical charge of mannoproteins is modified. In the pH range of wine, mannoproteins carry negative charges and, as a consequence, they may establish electrostatic and ionic interactions with the other components of the wine [41], resulting in the formation of either soluble or insoluble complexes in a process that is strongly dependent on their net electrical charge and on the structure of their functional groups [42]. In the genus *Saccharomyces*, the glycan portion of mannoproteins is composed not only of neutral oligosaccharides containing mannose and N-acetylglucosamine, but also of acidic oligosaccharides containing mannosylphosphate, in quantities which vary from strain to strain [43]. This modification can change the properties and environment of the cell surface, since mannosylphosphate gives a net negative charge to cell wall mannoproteins. For other yeasts, a different composition of the glycan portion of mannoproteins has been described: the oligosaccharides of *Schizosaccharomyces pombe* and *Kluyveromyces lactis* seem to contain galactose and N-acetylglucosamine, respectively, but not mannosylphosphate, whereas the

oligosaccharides of *Kloeckera brevis* and *Candida albicans* seem to contain as much mannosylphosphate as oligosaccharides of S. cerevisiae [44].

Mannoprotein from yeast was reported to be an effective bio-emulsifier; spent yeast from the manufacture of wine was demonstrated to be a possible source for large-scale production [45-47]. Future studies in this field could evaluate the biosurfactant properties of different yeast mannoproteins and their potential use in the food industry.

Mannoproteins are partially water-soluble components, released by the action of ß-1,3 glucanases during and, above all, after alcoholic fermentation [48]. Contact time, temperature, and agitation of the yeast biomass promote their enzymatic release [49]. ß-1,3 glucanases exhibit activity during yeast growth (wine fermentation), as well as in the presence of resting yeast cells (aging on lees). Production and release of mannoproteins seem to depend on the specific yeast strain [50], as well as the nutritional conditions [36].

Some studies reported that mannoproteins were able to give adsorption of ochratoxin A (OTA).

Ochratoxin A (OTA) is a dangerous fungal secondary metabolite; this mycotoxin has been reported in grapes, grape juices and wines [51]. Some studies reported that, during fermentation, three different strains of S. cerevisiae were able to decrease OTA added by as much as 21% [52]. Various decontamination procedures for the removal of OTA using yeasts [53-55], yeast cell walls [56-57], or yeast cell wall extracts [58] have been developed. Mannoproteins seem to play a considerable role in OTA adsorption, due to the mycotoxin-binding capacity reported for modified mannanoligosaccharide derived from the cell wall of S. cerevisiae [59,60]; moreover, the spontaneous nature of the OTA adsorption on yeast cell walls has been recently demonstrated.

Accordingly, mannoproteins may be used like a sponge, sequestering OTA in grape juices and wines.

Remarkable differences in the in vitro binding activity of wine yeasts towards OTA have been reported [36], which may be explained by the different mannosylphosphate content in the mannoproteins of each wine yeast.

In addition, it has been demonstrated that it is possible to greatly reduce the OTA content of grape must during winemaking by using expressly selected wine yeasts [61]. This has become more interesting since the decision of the European Community that wines produced from the 2005 harvest onwards must respect the maximum limit of 2.0 ppb [62].

Parietal yeast mannoproteins have been associated with stimulation of malolactic bacteria growth in wine [63]. This could be due to the adsorption of the medium chain fatty acids synthesized by Saccharomyces [64]. These compounds have been shown to inhibit bacterial growth and, therefore, their removal improves malolactic fermentation.

Moreover, malolactic bacteria are able to hydrolyse mannoproteins, thus enhancing the nutritional content of the medium and also stimulating their activity [65].

Malolactic fermentation consists of the conversion of L-malate to L-lactate and carbon dioxide, and plays an important role in winemaking because, besides lowering total acidity, it is usually believed to improve the biological stability and the sensory properties of the wines where it occurs.

Using mannoproteins, it is possible to prevent the tartrate salt insolubilization and precipitation of tartaric acid salt in the course of winemaking [36]. It has been shown that mannoproteins can effectively inhibit the crystallization of tartrate salt by lowering the crystallization temperature [36]. The crystal seeding process is slowed down by

highly glycosylated mannoproteins with molecular weights between 30 and 50 kDa, which improve tartaric stability.

Some studies reported that a polysaccharide active in promoting the stability of wine has been isolated and characterized from its total colloidal fraction [36]: it seems to be a high mass mannoprotein with a molecular weight of 420 kDa, present in a very low concentration in wine, 0.007% of total polysaccharides, which derives from fermenting yeasts. This glycoprotein seems to be a haze-protective factor [36]. The improvement of the wine's thermal stability by means of lees, is due neither to the removal of the unstable protein fractions, nor to the proteolytic activities present in yeasts, but rather to the addition of yeast mannoproteins [36]. Improvement in the protein stability of white wines during barrel aging on the lees is a well-known phenomenon.

It has been shown [36] that wine clarification and stabilization processes exert a negative influence upon sensory properties when the rate of eliminated macromolecules reaches 30%. When the macromolecule content of the wine is reduced by means of filtration, losses of colour intensity, aroma, and flavour are observed; intensity of aroma and persistence of flavour are reduced. Aroma stabilization is dependent on the hydrophobicity of the aroma compounds, and the protein component of the mannoproteins is important for overall aroma stabilization [36]. Interactions between mannoproteins and aromatic compounds can lead to modifications of volatility and aromatic intensity of wines; in this case, mannoproteins are free to interact and to fortify the existing aroma components.

In conclusion, it appears that parietal mannoproteins can play a very important role during the winemaking process and they can induce chemical, sensorial and health advantages, by improving wine quality.

The enological functions of mannoproteins are strictly related to their glycoprotein structure but there are very few studies in literature about their structural characterization.

1.2c Odorous metabolites in cheeses

Cheese flavour development is thought to occur through a complex series of biochemical reactions.

During the ripening process, the fat and proteins are partially hydrolysed to produce precursor compounds which, together with sugar, are converted into flavor components of the particular cheese variety.

The flavour of fresh cheese, which is ready to be eaten immediately after manufacture is mainly the result of the action of the starter bacteria, while the flavour of maturated cheeses is the result of the interaction of starter bacteria, enzymes from milk, enzymes from rennet and accompanying lipases and secondary flora [66,67].

Lipolysis is an important biochemical event occurring during cheese ripening and has been studied quite extensively in varieties such as Blue and hard Italian cheeses where lipolysis reaches high levels and is a major pathway for flavour generation. However, in the case of cheeses such as Cheddar and Gouda, in which levels of lipolysis are moderate during ripening, the contribution of lipolytic end products to cheese quality and flavour have received relatively little attention.

FFA are important precursors of catabolic reactions, which produce compounds that are volatile and contribute to flavour; however, these catabolic reactions are not thoroughly understood [68] (figure A).

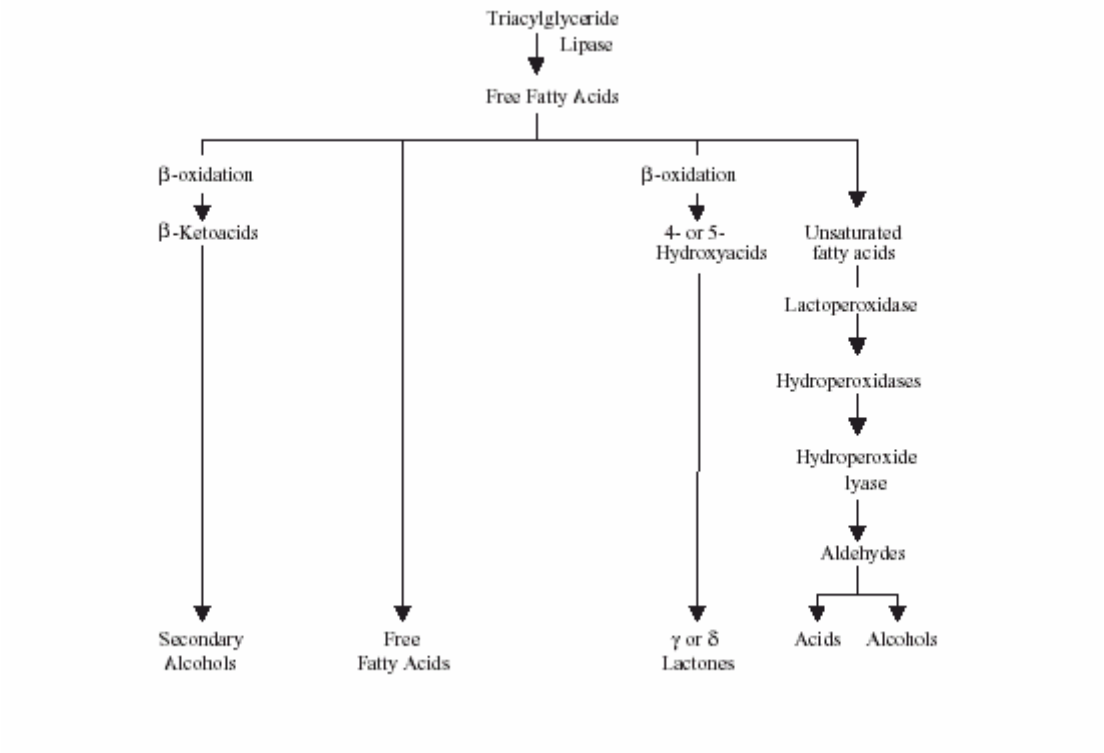

Figure A Catabolism of free fatty acids [69].

It is well established that milk fat is essential for the development of correct flavour in cheese during ripening. This was demonstrated in studies with cheeses made from skim milk, or milk in which milk fat had been replaced by other lipids; such cheeses did not develop correct flavour [70].

Lipids present in foods may undergo oxidative or hydrolytic degradation. Polyunsaturated fatty acids are especially prone to oxidation, which leads to the formation of various unsaturated aldehydes that are strongly flavoured and result in the flavour defect due to oxidative rancidity [71].

Lipid oxidation does not occur to a significant extent in cheese, probably because of its low redox potential [72] and the presence of natural antioxidants (e.g., vitamin E) [73]; its contribution to cheese flavour development is considered to be of little importance [66].

However, enzymatic hydrolysis of triacylglycerides (lipolysis) is essential to flavour development in some cheese varieties [66].

Lipolysis in cheese is due to the presence of lipolytic enzymes, which are hydrolases that cleave the ester linkage between a fatty acid and the glycerol core of the triacylglyceride, by producing FFA, and mono- and diacylglycerides [74]. Lipolytic enzymes may be classified as esterases or lipases, which are distinguished according to three main characteristics: (1) length of the hydrolysed acyl ester chain, (2) physico-chemical nature of the substrate and (3) enzymatic kinetics. Esterases hydrolyse acyl ester chains between 2 and 8 carbon atoms in length, while lipases hydrolyse these acyl ester chains of 10 or more carbon atoms. Esterases hydrolyse soluble substrates in aqueous solutions while lipases hydrolyse emulsified substrates.

The enzymatickin etics of esterases and lipases also differ; esterases have classical Michaelis–Menten type kinetics while lipases, since they are activated only in the presence of a hydrophobic/hydrophilic interface, display interfacial Michaelis–Menten type kinetics [66]. Unfortunately, the terms ‘‘esterases’’ and ‘‘lipases’’ are often used interchangeably in the scientific literature [66].

FFA are released upon lipolysis and can contribute directly to cheese flavour, especially short- and intermediate chain FFA [66].

Lipases in cheese originate from six possible sources: (1) milk, (2) rennet preparation (rennet paste), (3) starter, (4) adjunct starter, (5) non-starter bacteria and, possibly, (6) their addition as exogenous lipases [66].

Milk contains a very potent indigenous lipoprotein lipase (LPL), which normally never reaches its full activity in milk [66].

Commercial rennets are normally free from lipolytic activity. However rennet paste, used in the manufacture of some hard Italian varieties (e.g., Provolone, Romano),

contains the lipase, pregastric esterase (PGE) [75]. Rennet paste is prepared from the abomasa of calves, kids or lambs slaughtered after suckling. The abomasum is partially dried and ground into a paste, which is slurried in milk before being added to cheese milk.

Lipases and esterases of lactic acid bacteria (LAB) appear to be the principal lipolytic agents in several cheeses made from pasteurized milk [66]. Early studies on the role of LAB and lipolysis [66] concluded that partially hydrolysed milk fat was a better substrate for lipolysis by starter bacteria than unhydrolysed milk fat. To hydrolyse milk fat in milk and cheese, LAB possess esterolytic/lipolytic enzymes capable of hydrolyzing a range of esters of FFA, tri-, di-, and monoacylglyceride substrates [76,77]. Despite the presence of these enzymes, LAB, especially Lactococcus and Lactobacillus spp. are generally considered to be weakly lipolytic in comparison to species such as Pseudomonas, Acinetobacter and Flavobacterium [78]. However, because of their high presence in cheese over an extended ripening period, LAB are considered likely to be responsible for the liberation of significant levels of FFA.

Amino acid catabolism by lactic acid bacteria (LAB) is a a slow process that occurs mainly during cheese ripening. In LAB, the main route for amino acid conversion into aroma compounds is initiated by a transamination reaction that transforms amino acids into α-keto acids. The α-keto acids are then converted into various aromatic compounds such as carboxylic acids, aldehydes and alcohols and non aromatic hydroxy acids (figure B) [79].

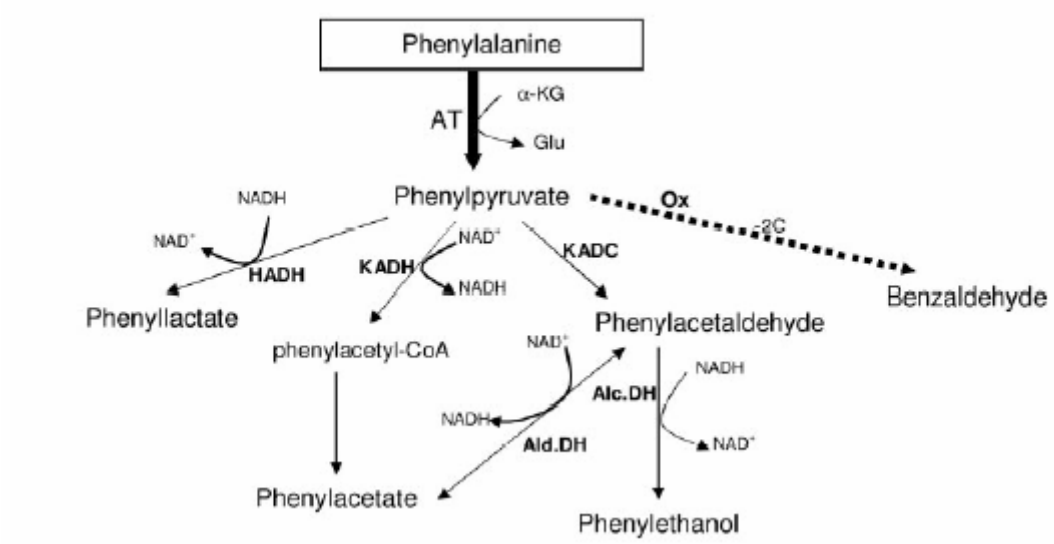

Figure B Phenylalanine catabolism pathways in *Lactococcus lactis*. _-KG, _-ketoglutarate; Glu, glutamate; AT, aminotransferases; HADH, hydroxy acid dehydrogenase; KADH, ketoacid dehydrogenase; KADC, ketoacid decarboxylase; Alc.DH, alcohol dehydrogenase; Ald.DH, aldehyde dehydrogenase; CoA, coenzyme A; Ox, chemical oxidation; _2C, loss of 2 carbons [79].

The production of these compounds primarily depends on the enzyme content of the strains. For example, some LAB strains produce aldehydes and alcohols as a result of an α-keto acid decarboxylase activity, while some other strains mainly produce carboxylic acids and hydroxy acids.The production of these metabolites is also affected by environmental factors such as pH and NaCl concentration, as these factors affect the enzyme activities. Because of the low buffer strength of cytoplasm in comparison with oxidoreduction potential (Eh), extracellular Eh is also an environmental factor that affects bacterial metabolism [79].

Amino acid catabolism is mainly initiated by a transamination reaction, which requires the presence of an α-keto acid as the amino group acceptor. The production of an α-keto acid acceptor by LAB often limits amino acid catabolism, but strains exhibiting glutamate dehydrogenase (GDH) activity are capable of producing α-ketoglutarate (α-

KG) from glutamate (Glu) and therefore are capable of degrading amino acids in a reaction medium containing Glu [80].

Glycolysis in natural cheese principally concerns metabolism of lactose to L-lactic acid and its subsequent conversion to D-lactic acid or its degradation to acetate, propionate or, in certain cases, to butyrate.

The fermentation of lactose into lactic acid by lactic acid bacteria can be considered another essential primary reaction in the manufacture of all cheese varieties.

The reduced pH of cheese curd, which reaches 4.5 to 5.2, depending on the variety, affects at least the following characteristics of curd and cheese: syneresis (and hence cheese composition), retention of calcium (which affects cheese texture), retention and activity of coagulant (which influences the extent and type of proteolysis during ripening), the growth of contaminating bacteria. Most (98%) of the lactose in milk is removed in the whey during cheesemaking, either as lactose or lactic acid. The residual lactose in cheese curd is metabolized during the early stages of ripening. During ripening lactic acid is also altered, mainly through the action of nonstarter bacteria. The principal changes are (1) conversion of L-lactate into D-lactate so that a racemic mixture exists in most cheeses at the end of ripening; (2) in Swiss-type cheeses, L-lactate is metabolized to propionate, acetate, and CO_2, which are responsible for eye formation and contribute to typical flavor; (3) in surface mold, and probably in surface bacterially ripened cheese, lactate is metabolized to CO_2 and H_2O, which contributes to the increase in pH characteristic of such cheeses and that is responsible for textural changes, (4) in Cheddar and Dutch-type cheeses, some lactate may be oxidized into acetate by Pediococci. Cheese contains a low level of citrate, metabolism of which by Streptococcus diacetylactis leads to the production of diacetyl, which contributes to the

flavor and is responsible for the limited eye formation characteristic of mentioned cheeses [81].

Alcohols in cheeses can derive from enzymatic reactions on lactose with production of lactic acid and other metabolites.

The aldehydes can also derive from non enzymatic degradation (Strecker reaction) of amino acids.

Generally terpene compounds in cheeses derive from specific animal feeding.

Some studies reported that the alpine cheeses contained, on average, about 3 times as much limonene and 4 times as much nerol, which on the contrary was not detected in any of the lowland cheeses. These studies can be useful where cheese producers want to claim particular naming rights for cheeses from a small specified area, as it is the case for wines [82].

2. MATERIALS AND METHODS

For the determination of odorous metabolites and their precursors, and for the analysis of mannoproteins, a combined use of extraction, purification and mass spectrometric techniques was used.

Some technical indications of the main techniques used are reported below.

2.1. Solid phase extraction

The main steps of the SPE procedure are: conditioning, sample application, washing, elution

In these steps the following system parameters have been considered: nature of sorbent, of rinsing and elution solvents, dimensions of the bed (*V*M or bed holdup volume and *N*, number of plates of the bed), volume of sample to load, *V*L, volume of rinsing solvent, *V*RS, and volume of elution solvent, *V*E.

The following key parameters have been considered: maximum volume of sample and of rinsing solvent that can be passed through the SPE bed without losses of analyte, VLmax +VRSmax (VLmax ≤Vbreakthrough), minimum volume of rinsing solvent that should be passed to completely eliminate interferences, VminRS, minimum volume of elution solvent that should be passed to completely elute the analyte, VminE, maximum volume of elution solvent that can be passed without eluting additional interferences, VmaxE.

The isolation will be successful if a sufficient sample volume can be loaded and at least one of the two following conditions is fulfilled:

1.VminRS<VmaxRS Elimination of interferent species with no analyte loss.

2. VminE<VmaxE Complete recovery of the analyte without elution of additional interferences

Breakthrough curves can be also considered.

In order to give an example, calculated breakthrough curves for a SPE device with: *VM* = 0.42 mL ; Ks = 100; N = 5 (curve A); 20 (curve B) and 100 (curve C) plate/cm are the following:

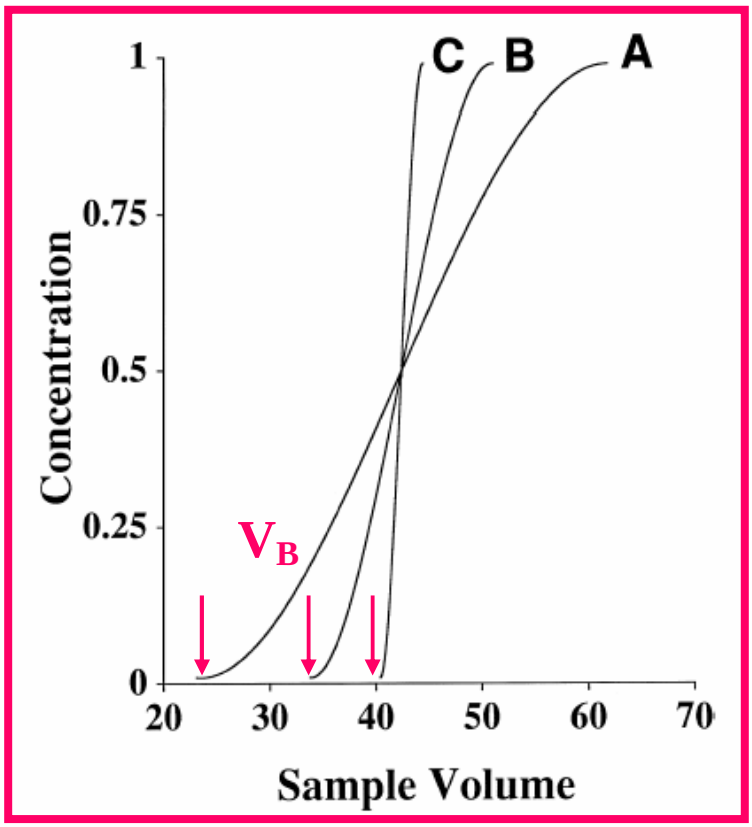

Figure C Example of breakthrough curves for a SPE device with: *VM* = 0.42 mL ; Ks = 100; N = 5 (curve A); 20 (curve B) and 100 (curve C) plate/cm.

$$V_B = (1 + k_s)V_M\left(1 - \frac{2.3}{\sqrt{N}}\right) \quad (1)$$

or

$$V_B = \frac{1}{\sqrt{a_0 + a_1/N + a_2/N^2}}(1 + k_s)V_M \quad (2)$$

VB = breakthrough volume at 1% breakthrough level (interparticle velocity affects VB; the the largest VB is obtained at the lowest sample flow rate; *VM* = bed holdup volume, Ks = chromatographic retention factor in the liquid sample loaded onto SPE, N = number of plates ; *a0*, *a1* , *a2* are coefficient characteristic of the breakthrough level. Equation 2 applies to systems with a low number of plates *[83]*.

The required elution volume VE for 99% recovery of the analyte, is given by:

$$V_E = (1 + k_E) V_M \left(1 + \frac{2.3}{\sqrt{N}}\right)$$

Where *K*E is the retention factor of the analyte in the elution solvent; small sorbent bed (minimizing VM) and a strong solvent (*K*E <3 and ideally 0) minimizes VE.

Large values of *N* provide sharper desorption front profiles and require a smaller elution volume to quantitatively recover the analyte from the sorbent trap.

The ratio VB/VE gives the pre-concentration factor.

To select and optimize the sample processing conditions for SPE requires knowledge of the retention factors of the analytes under different mobile phase conditions corresponding to sample application, rinsing (matrix simplification) and elution. An indirect measure of the retention factor is the solid–liquid distribution coefficient K (K = K ϕ, ϕ is the phase ratio). This parameter can be easily measured and can provide useful information about the behavior of analytes and interferences during sample application, rinsing and elution.

A simple and practical approach has been recently proposed [84,85]:

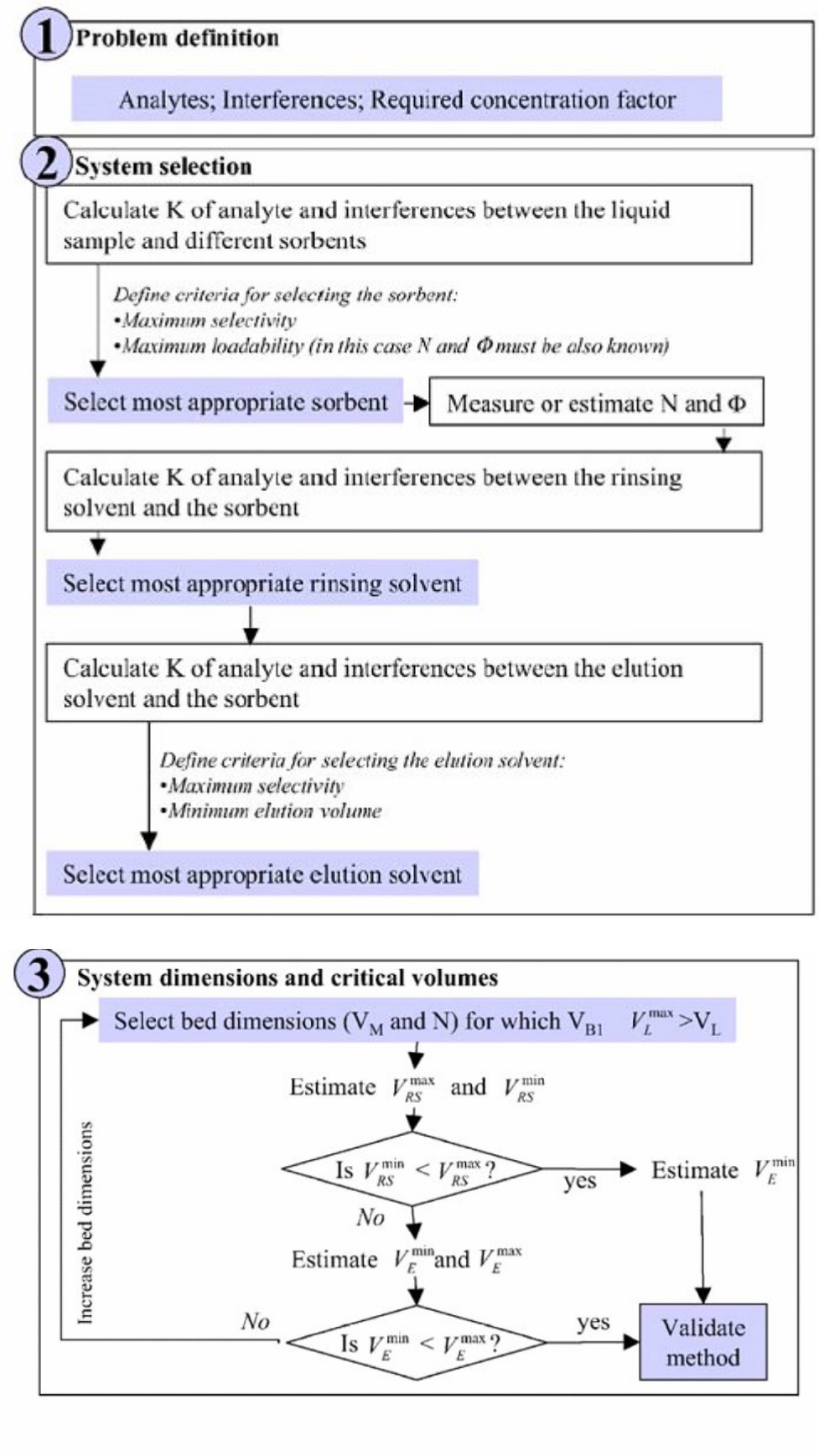

Figure D Proposed algorithm for SPE method optimization [84,85].

2.2. Solid Phase Microextraction

SPME is based on multiphase equilibration process.

In an ideal three-phase system, fiber coating, gas phase or headspace, and a homogeneous matrix, such as pure water, during the sampling period, the analytes migrate among the three phases until equilibrium is achieved.

The mass balance relationship is:

Where C0 is the initial concentration of analyte in the matrix Cc∞ Ch∞ and Cs∞ are the equilibrium or final concentrations of analyte in the coating, headspace and sample, Vc, Vh, Vs are the volumes of the coating, headspace

and sample, respectively.

$$C_0 V_s = C_c^{\infty} V_c + C_h^{\infty} V_h + C_s^{\infty} V_s \qquad (1)$$

The coating /headspace and headspace/sample distribution coefficient can be defined as: (2) Kch= Cc∞ / Ch∞, (3) Khs= Ch∞ / Cs∞ The mass of the analyte absorbed on or in the coating is given by: (4) n=Cc∞ Vc

Combining eqs. (1) – (4), n can be expressed as:

$$n = \frac{K_{ch} K_{hs} V_c C_0 V_s}{K_{ch} K_{hs} V + K_{hs} V_h + V_s} \qquad (5)$$

In addition the coating/sample distribution coefficient is:

$$K_{cs} = K_{ch} K_{hs} \qquad (6)$$

and Eq. (5), therefore, can be simplified as:

$$n = \frac{K_{cs} V_c C_0 V_s}{K_{cs} V_c + K_{hs} V_h + V_s} \qquad (7)$$

It is significant that Eq. 7 states that the amount of analyte extracted is independent of the location of the fiber in the system [85].

2.3. Affinity chromatography

According to the International Union of Pure and Applied Chemistry, affinity chromatography is defined as a liquid chromatographic technique that makes use of a "biological interaction" for the separation and analysis of specific analytes within a sample. Examples of these interactions include the binding of an enzyme with an inhibitor or of an antibody with an antigen. Lectins are a class of ligands that have been used for the direct detection of clinical analytes by affinity chromatography. The lectins are non-immune system proteins that have the ability to recognize and bind certain types of carbohydrate residues. The lectin concanavalin A, binds to α-D-mannose and α-D-glucose residues [86].

Affinity chromatography can be capable of purifying a single protein from a complex mixture in a single step. However, even if this theoretical ideal is not achieved, the degree of purification is commonly efficient.

The procedure for affinity chromatography of proteins is similar to that for the other types of liquid chromatography. The matrix is packed into a column in a buffer that will be optimal for enzyme-ligand binding. Thus the buffer must contain any co-factors such as metal ions that are needed for binding. Usually the buffer has a fairly high ionic strength to minimize non-specific binding of other proteins to the ligand. The sample is applied and washed through the column. Ideally, only the molecule of interest should bind. The molecule of interest can then be eluted specifically by the addition of a relatively high concentration of competitive molecule or, failing this, by changing the pH and/or ionic strength to disrupt the "biological interaction".

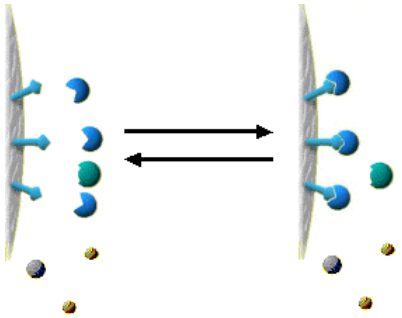

Figure E Reversible binding between receptor and ligand in affinity chromatography. The unbound molecules are washed away.

2.4. Mass spectrometric techniques

Mass spectrometry (MS)-based methods have drawn more and more public attention in recent years in various fields including food as well. New European laws have increased the standards for human health, safety and quality of food products.

The quality standards include the re-assessment of the legal action levels, e.g. maximum residue limits (MRLs) of pesticides and veterinary drugs, which are typically lower than the previous ones. This in turn has promoted the development of more powerful, sensitive analytical methods to meet the requirements in complex samples such as food [87].

In this context liquid chromatography tandem mass spectrometry (LC-MS/MS) mode is the most widely used technique for the quantitation of polar contaminant residues in food. High-resolution MS techniques such as high-resolution time-of-flight MS (TOF-MS), have been applied mainly for structure elucidation or confirmation purposes. GC-MS and GC-MS/MS are applied analogously to GC-amenable compounds [87].

In the study of food proteins, accurate protein structural analysis is necessary because of the fact that nucleotide sequencing alone is of limited analytical value if not combined with relevant information regarding the specific protein expressed and the occurrence of

phosphorylation, glycosylation and disulphide bridges, and with the modification induced by the technological treatment [88].

New mass spectrometric techniques, used alone or to complement the traditional molecular-based techniques are an efficient tool for protein and peptide analysis in complex mixtures, such as food matrices.

All mass spectrometric techniques allow to detect molecules in form of ions, giving values of molecular mass (m/z, ratio of mass to charge).

Matrix-assisted Laser Desorptio Ionization (MALDI) with Electrospray ionisation (ESI) are the most important ionisation methods for non-volatile molecules, high molecular weight compounds, in particular peptides, proteins, oligosaccharides and oligonucleotides.

A number of chemical and physical pathway have been suggested for MALDI ion formation, including gas-phase photoionization, ion-molecule reactions, disproportionation, excited-state proton transfer, energy pooling, thermal ionisation, and desorption of preformed ions.

In ESI-MS, a dilute solution of analyte is pumped through a capillary at a very low flow rate (0.1-10 ul/min); a high voltage is applied to the capillary. This voltage can be either negative or positive, depending on the analytes chosen. The applied voltage provides the electric-field gradient required to produce charge separation at the surface of the liquid.

For volatile compounds other kinds of ionisation are used; the electron ionisation (EI) produces ions bombarding molecule, the resulting molecule is an ion and has a charge (usually +1).

2.5. Mass spectrometric techniques and analysis of peptides and proteins

Protein analysis can be divided into two basic categories: mass analysis and amino acid sequencing.

Sequence information is not a priori determined by measurement of either a peptide or a protein. Consequently enzymatic strategies have been coupled with mass measurement: proteins are digested with a protease (usually trypsin) and studied by MS.

By means of MS/MS the original peptide fragments are fragmented. The resulting MS peaks represent a protein sequence.

2.6. Materials

The solvents and reagents used for the analysis were purchased by Sigma (Aldrich, Bornem, Belgio).

The SPE columns Strata X (30 mg, 1 ml) and Nexus (30 mg, 1 cc) were purchased respectively by Phenomenex (USA) and by Varian (USA).

The SPME holder and fibers (PDMS, CAR/PDMS, CAR/DVB/PDMS) were purchased by Supelco (Aldrich, Bornem, Belgio). The TMS-derivatives of terpene glycosides were obtained with N,O-Bis(trimethyl-silyl)trifluoroacetamide 1% chlorotrimethylsilane (Sigma).

Concanavalin A was purchased by Sigma (Aldrich, Bornem, Belgio).

2.7. Analytical methods used for the aromatic characterization of wines

In order to obtain more complete data as possible for the aromatic composition of the typical wines, the analytical approach was articulated through different strategies of extraction and concentration (liquid/liquid extraction; solid phase extraction; solid phase micro-extraction; static headspace analysis).

The liquid extraction was carried out with 2.5 ml of dichloromethane and 50 ml of wine on a vortex for 1 hour.

The solid phase extraction took place by diluting 1/1 the wine sample with water; loading was effected with 12 ml of diluted wine on the StrataX SPE and 24 ml on Nexus SPE. The elution was carried out with 400 µl of dichloromethane in both cases.

The SPME was carried out with the following conditions: the fibers were immersed in the headspace (HS) of the samples using 200 ml of wine until equilibrium conditions. Thermal desorption of the analytes from the fiber inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250 °C during 1 minute.

For the analysis of static headspace an autosampler Agilent 7694E was used.

2.8. Analytical methods used for odorous molecules and their precursors in grapes

Grapes in good sanitary conditions from regione Campania (Italy) were collected and stored at –20°C until analysed.

For the analysis with static headspace 10 ml of grape juice were mixed with 2 g of NaCL and were introduced in vial for headspace autosampler Agilent 7694E.

The SPME analysis was carried out on 100 mL of grape juice with 15 g of NaCl.

Terpene glycosides were extracted with fractionated extraction with C18 cartridge (Baker; 500 mg/3mL) starting from 15 mL of grape juice. The step of washing was effected with 15 ml of water and with 25 ml of dichloromethane for eluting free odorous compounds. The elution of glycosides was carried out in more than 4 steps with 10 ml of methanol respectively at 20%, 30%, 40% in water for the first three steps, and 100% methanol in the final step.

The eluted fractions were dried; TMS-derivatives were obtained by means of reaction of derivatization with N,O-Bis(trimethyl-silyl)trifluoroacetamide 1% chlorotrimethylsilane (Sigma)at 60°C for 45 minutes.

2.9. Analytical methods used for the aromatic characterization of Mozzarella di Bufala Campana cheeses

The aromatic characterization of Mozzarella di Bufala Campana cheeses was carried out by means of HS-SPME-GC/MS analysis on 100 ml of omogeneized samples mixed with 20 g of NaCl.

The analysis of short chain FFA was carried out on 50 g of sample, through liquid extraction by using acetonitrile (20 ml); GC/MS analysis was effected with direct injection.

2.10. Analytical methods used for the extraction and characterization of mannoproteins

Mannoproteins were extracted with affinity chromatography with concanavalin A.

Concanavalin A requires Mn2+ and Ca2+ ions for carbohydrate binding.

The chromatographic procedure used was: 1. Pre-washing of column with 5 column volumes of wash solution (1 M NaCl, 5 mM MgCl2, 5 mM MnCl2, and 5 mM CaCl2). 2. equilibation of column in the buffer 20 mM Tris, pH 7.4, containing 0.5 M NaCl. 3. Loading of 50 ml of wine sample after dialysis. 4. Washing of the column with equilibration buffer. The elution was carried out by means of mannose 1 M.

In order to analyse glycosyl-residues bound to the proteins, methanolysis of mannoproteins was performed with anhydrous methanol 1 M at 100°C for 16 hours.

Trimethylsilylation of glycosyl residues was carried out with N,O-Bis(trimethyl-silyl)trifluoroacetamide 1% chlorotrimethylsilane (Sigma) at 80°C for 20 minutes.

2.11. Mass spectrometric analysis

For gas chromatography-mass spectrometric analysis, all samples were analysed with an HP 6890 coupled to a 5973N quadrupole HP mass spectrometer. The gas chromatograph was equipped with an HP-5 ms capillary column (30m x 0.32 mm ID) and the carrier gas used was helium.

For the analysis of free compounds, the GC oven temperature was programmed from 40°C (held for 7 minutes) at 5 °C/min to 180 °C. The mass spectrometer was operated in electron mode (EI, 70 eV) and the masses were scanned over an m/z range of 45-350 amu. In other cases a method SIM was used (for terpene compounds m/z 93, 12, 136).

The identification of odorous components was effected by NIST library and/or by comparison with spectra and retention times of standards. The discriminant analysis was effected by means of SPSS statistical software (version 12.0).

For the analysis of glycoside compounds the GC oven temperature was programmed from 120°C (held for 4 minutes) at 3 °C/min to 300 °C. The mass spectrometer was operated in electron mode (EI, 70 eV) and the masses were scanned over an m/z range of 45-600 amu.

The LC/MS analyses were carried out by means of a LC/MS instrumentation (HP1100-MSD, Agilent Technologies) with single quadruple using C18 column (2.1 mm x 250 mm) and a gradient from 5% Acetonitrile (0,2% formic acid, buffer A) and 95% water (0,2% formic acid, buffer B) to 70% buffer B in 45 minutes, with a flow of 0,2 mL/min.MALDI-TOF spectra were recorded in positive-ion mode, using an Applied Biosystems Voyager DE-PRO spectrometer.

3. Results

3.1. Aromatic characterization of autochthonous non aromatic grapes

In order to indicate the methodological approach adopted for the varietal characterization of typical grapes and wines, detailed results obtained for Falanghina grapes are reported. Our methodological approach started from grapes, with the identification of terpenoid, norisoprenoid compounds and aroma precursors (terpene glycosydes). Preliminary results obtained for some other white and red grapes and wines are also reported.

3.1.1. Autochthonous white grapes

3.1.1a Falanghina grapes

Terpenes and norisoprenoids detected in Falanghina grapes are indicated in figure 1 where the TIC chromatogram obtained for a Falanghina grape sample through static headspace-GC/MS analysis (SCAN method) is reported. The presence of two norisoprenoids were also observed: β-damascenone, with floreal and fruit odorous nuances and α-ionone with violet odorous nuances.

In table 1 varietal odorous molecules for Falanghina grape samples from two geographical areas (BN and Campi Flegrei) are reported. Some terpenes, myrcene, furanlinalol oxide, geraniol, 4-carene, resulted detectable in Falanghina grapes and not in Coda di Volpe grapes. Among Falanghina grape samples from different geographical areas (Benevento and Campi Flegrei) only quantitative differences were observed for these compounds (the presence of all terpenes identified, limonene, geraniol, linalool, myrcene, 4-carene, cis-linalool oxide, alpha-terpineol, were higher in samples from Beneventane area in comparison with samples from Campi Flegrei, while the quantities of norisoprenoid compounds (α-ionone, β-damascenone) were similar; in figure 2 the SIM chromatograms obtained by means of HS/SPME-GC/MS analysis for a sample of Falanghina grape from Benevento area (a) and from Campi Flegrei (b) are shown.

In figure 3 the TIC chromatograms obtained by means of headspace-SPME-GC/MS analysis of Falanghina grape juice is shown. Peaks with major area correspond to C6 compounds (hexanal, 1-hexanol, 2-hexenal) with odorous herbaceus descriptor, the contribution of which to the aromatic profile is considered dominant in non aromatic grapes in comparison with aromatic varieties. Furthermore, their formation seems to increase with the presence of oxygen and of lipoxygenase-like enzymatic systems [89].
Other odorous molecules detected for Falanghina samples were ethyl hexanoate, benzeneacetaldheyde, nonanal, phenyl ethyl alcohol, ethyl octanoate, decanal, ethyl decanoate, terpenes, some compounds considered terpene derivatives, norisoprenoids.
For the analysis of terpene glycosides of Falanghina grapes, the fractionated extraction (obtained through stepwise elution at 20%, 30%, 40%, 100% methanol) with C18 SPE was needed in order to reduce interferences which create difficulties for non aromatic grapes where the terpene compounds are in lower concentration in comparison with aromatic varieties.
In order to obtain an evidence of identity for terpene glycosides information was obtained by means of a combined use of mass spectrometric techniques.
At first the identification of glycosides was effected through GC/MS analysis of TMS-derivatives considering fragmentation spectra for TMS-derivatives of these compounds reported in literature [90,91]; further indications were obtained through LC/ESI/MS analysis in both positive and negative ion mode;
In figure 4 the TIC Chromatogram obtained by means of GC/MS analysis of an extract eluted in the elution step with 100% methanol is showed.
The extracts containing the native terpene glycosides were also analysed by LC/ESI/MS; in figure 5 the TIC chromatogram obtained in negative ion mode is shown for an extract of Falanghina grape.
In figures 6a'), 6 b') and 6c') signals obtained in negative ion mode ($[M-H]^-$) are indicated respectively for linalyl glucoside (Mw 316 Da, tr=11.5 min), for linalyl arabinosylglucoside (Mw 448 Da, tr=24.6 min), for linalyl rhamnosylglucoside (Mw 462 Da, tr=26.7 min). In figures 6a'', 6b'') 6c'') corresponding peaks obtained in positive ion mode ($[M+H]^+$) are shown. The best results were obtained in negative ion mode.

In table 2 a comparison of composition in terpene glycosides is shown for different grapes.

3.1.1b Some other white grapes

TIC chromatograms obtained for other different non aromatic white grapes (Fiano, Greco, Coda di Volpe, Asprinio) by means of HS-SPME-GC/MS analysis are shown in figures 7-10.

Preliminary results obtained for the terpene composition of different autochthonous grapes of region Campania are indicated in table 3.

Differences of terpene composition were oserved: some compounds were present in Falanghina and Fiano grapes but were not present in Coda di Volpe, Greco, Asprinio grapes. These results confirm the strict dependence of terpene composition on grape variety.

3.2. Aromatic characterization of wines

The aromatic characterization of a wine requires qualitative and quantitative determination of a large number of different components (varietal and non varietal); for the identification of odorous molecules of wines different methods of extration and concentration were used (headspace-SPME carried out with different fibers; SPE with different support; liquid/liquid extraction, ecc.).

3.2.1. Falanghina wine

Dominant terpenes and norisoprenoids observed in Falanghina grapes were detected also in all samples of Falanghina wines analysed (figure 2).

In wine samples also the presence of all terpenes identified (limonene, geraniol, linalool, myrcene, 4-carene, cis-linalool oxide, alpha-terpineol) is higher in samples from Benevento area than in samples from Campi Flegrei (figure 11).

Odorous molecules identified in Falanghina wines are indicated in table 4.

Terpene glycosides identified in grapes were identified in wine also.

3.2.2. Discriminant analysis: efficient tool for the differentiation of typical wines

Discriminant analysis was tentatively used for the differentiation of typical Falanghina wines from others.

The application of discriminant analysis for some samples of Falanghina wines (group 3) and other two typical wines (Lacryma Christi and Greco di Tufo)(group 1, group 2) is indicated in figure 12 which provided projections of samples on the first two discriminant axes; proximities in the plane represent proximities in the subspace generated by the first two discriminant functions; as the first two components completely reconstructed original data, proximities in the plane also represent proximities among original samples; the factorial plain is defined by the two discriminant functions whose standardized coefficients are indicated in table 5, other synthetic information about this representation is indicated in table 6. Discriminant analysis was effected by using the quantitative ratios measured for some representative aroma compounds in aromatic bouquet for Falanghina wine [92]. The identified descriptors for the analysed samples were the following: ethyl butanoate, 1-butanol, 3-methyl acetate, ethyl hexanoate, ethyl butandioate, ethyl octanoate, ethyl decanoate, ethyl succinate, phenyl ethyl acetate, phenyl ethyl alcohol.

3.2.3. Modification of the terpene composition during the wine shelf-life

During the winemaking process terpene glycosides can be hydroxylated by means of acid hydrolysis or enzyme hydrolysis and consequently the concentration of terpenols can increase from some micrograms per liter to some milligrams per liter. At the same time some other slow modifications of terpene composition can occur, for example, if the wine, after the winemaking process, is stored at high temperatures and in light, some inter-conversions of terpene compounds could become evident:

Linalool and geraniol can be converted into alpha-terpineol and the degradation of terpenes can give p-cymene as final product [93].

The occurrence of these structural modifications of terpenes can be proved with an experiment carried out at acid pH (1-3) and at high temperatures (60-80°C), on solutions of terpene standards.

After acid hydrolysis carried out at 80°C for 5 hours on solutions of terpene standards, limonene appeared very stable chemically; alpha-terpineol was relatively stable

chemically, however after acid hydrolysis from it some non-terpene products were observed (p-cymene), and in low quantities isoterpinolene was formed. Geraniol and linalool appeared unstable under acid hydrolysis conditions: they were converted in some other terpene compounds such as alpha-terpineol.

We observed that high temperatures and long storage times gave major convertion of terpenes into alpha-terpineol in some wine samples analysed. In samples stored at 35 °C only, p-Cymene had been detected after 1, 2, 3 months. During the storage period, alpha-ionone increased, which was derived from its hydroxylated bound.

Storage conditions had been proved critical to preserve varietal characteristics of typical wine.

3.2.4. Winemaking odorous markers

For some typical wines, dependence of aromatic composition and typical characteristics on winemaking process was studied.

A comparison in aromatic composition among Campi Flegrei and Benevento Falanghina wine samples fermented in barrique and in steel containers was effected.

Detected aromatic molecules released by barrique used for Falanghina wine were : 2-methoxy-phenol (guaiacol), aromatic descriptor=spices), 4-ethyl phenol (ink), 4-metyl guaiacol (medicinal), eugenol (cloves), vanilline (vanilla), acetovanillone (vanilla), butyrrolactone (butter), 5-ethyl furfurale, ethyl hexyl disulphide).

According to the analytical results the sensorial analysis indicated more complex aroma composition of samples fermented in barrique in comparison with samples fermented in steels, with nuances of vanilla, honey, spices; while samples fermented in steel containers showed floral and fruity nuances prevalently. In barrique lower concentrations of esters were formed and in total the quantity of aromatic molecules was higher than in fermentation in barrique (fermentation conditions in barrique were different from steel container, the wood, in fact, is a thermal insulation).

For samples fermented in barrique the sensorial analysis indicated that the typical aromatic characteristics shown by typicalness disciplinary were significantly modified.

Fiano wines with élévage in barriques also showed different sensorial qualities in comparison with samples with élévage in steels, with nuances of ripened fruits, while the élévage in steels produced mainly floral and fruity nuances.

Differences among Fiano samples aged in barriques (figure13) with different usage time were also observed. Studies of release potentialities carried out on different barriques showed significantly higher potentialities of release for new barriques in comparison with barriques used precedently.

The quantity of aromatic molecules released by barrique was strictly dependent on the entity of its usage.

3.3. Characterization of proteins with important enological functions present in wines (parietal mannoproteins of *Saccharomyces cerevisiae*)

Elaborated analytical strategies allowed to analyse mannoproteins extracted from different samples of wines.

In figure 14 a spectrum of an extract obtained with affinity chromatography for a Falanghina wine sample is shown.

Detected molecular weights were ranged between 10 kDa and 70 kDa, indicating a possible large heterogeneity of the composition in mannoproteins present in wine.

The observed heterogeneity led us up to separate mannoproteins extracted by means of HPLC analysis.

Chromatograms obtained by means of HPLC analysis of mannoproteins, from different samples of wines, showed an evident similarity, but variations of retention times and peak areas indicated quali-quantitative differences (figure 15).

The HPLC peaks were collected and analysed by means of MALDI-TOF-MS. The spectra corresponding to main peaks present in the HPLC chromatogram of wine extracts showed, in this case also, a large variability in the molecular weights.

The identification of mannoproteins extracted was effected through their tryptic digests and through comparison with data present in database and data obtained for standard mannoproteins extracted by yeasts.

In figure 16 spectrum obtained for a tryptic digest of mannoproteins extracted from wine is shown. Structural heterogeneity in the peptide sequences was observed. Sometimes mass differences corresponding to mannose residues were observed for peptide molecular weights detected.

Some peptide sequences were present simultaneously in more peaks of the HPLC chromatogram indicating that some mannoproteins in wine can differ only for the absence of a peptide sequence.

Analysis by means LC/MS/MS was also carried for some tryptic digests.

Peptide sequences identified in the wine samples analysed correspond to the mannoproteins indicated in table 7.

The presence of mannose bound to protein components was confirmed by GC/MS analysis of TMS-derivatives of glycosyl-residues after methanolysis (figure 17).

Residues of *Arabinose*, *Rhamnose*, *Fucose*, *Glucose* were also observed and their relative composition was variable in different samples.

3.4. Aromatic characterization of Mozzarella di Bufala Campana cheeses from different producers in region Campania

The aromatic characterization of Mozzarella di Bufala Campana cheeses was carried out on detectable odorous molecules present in headspace of analysed samples by means of HS-SPME-GC/MS analysis. In this way only compounds which gave major odorous impact were analysed. Mozzarella di Bufala Campana cheese samples from different producers within the Consortium showed differences in qualitative and quantitative composition of several odorous molecules: aldheydes, ketones, alcohols, hydrocarbons, lactones, esters, terpenes, with several different odorous descriptors (green, mushroom, floral, fruity, sweat, animal, hot milk etc.).

In table 8 a comparison of the composition in odorous molecules for samples from different producers is shown.

In the aroma profile of some samples terpene compounds (derived from specific animal feeding) were dominant (figure 18), while in some other samples esters (derived from action of esterases from starter) were dominant (figura 19) in the aroma profile of this typical cheese.

Mozzarella cheese show generally low FFA (free fatty acids) concentrations, which is associated with its mild flavour [94]; the FFA concentrations can play a key role for the flavour of this typical cheese.

The FFA composition of this typical cheese can differ a lot, the analysis of FFA carried out by gas chromatography-mass spectrometry showed a remarkable variability in the quantitative composition, with variation of concentrations from 2 to 50 folds between analysed samples.This large variability in FFA composition can be related to the specific characteristics of the starter used.

Results indicated that the aromatic profile of Mozzarella di Bufala Campana is not yet “standardized”, because of several variables related to the making process (milk origin, animal feeling, starter used,…).

Differences in FFA composition were also observed between liquid obtained after pressing and remaining pressed mozzarella cheese. The contribution of FFA with higher number of atoms of carbon increased in the pressed cheese in respect of liquid obtained after pressing, because of more polar characteristic of pressing liquid in comparison

with more idrophobic pressed matrix: different quantities of juice in mozzarella cheese could influence its flavour.

Results indicated evident differences in odorous molecules between different samples of Mozzarella di Bufala Campana which can depend on starter used and on animal feeding. The aromatic profile of this typical cheese is not “standardized”. Selected starters and adeguate feeding for animals can improve the sensorial quality of Mozzarella di Bufala Campana typical cheese.

4. Conclusions

The continuous evolution of new analytical technologies and scientific knowdledges leads up to introduce new analytical strategies aimed at obtaining key information about the food quality, which combined with conventional analytical parameters, can better describe the typicalness of typical food products. Mass spectrometric techniques, used alone or to complement the traditional molecular-based techniques are a necessary tool for structure elucidation and confirmation purposes in the analysis of several different metabolites present in foods; furthermore these techniques are instrumental for protein and peptide analysis in complex mixtures, such as food matrices, by giving information regarding the specific protein expressed and the possible occurrence of phosphorylation, glycosylation or disulphide bridges.

With regard to the results indicated in this study, for the aromatic characterization of typical non-aromatic wines, the analysis of varietal odorous molecules in grapes appeared an important preliminary step in order to obtain as complete analytical data as possible; because of the low quantities of varietal molecules, immediate analytical evidence of their presence was not always possible for wine samples; in several cases their complex aromatic profile led to co-eluting chromatographic peaks.

Terpenoid and norisoprenoid composition in the analysed autochthonous grapes of region Campania was strictly dependent on the grape variety (these compounds, present in free or non odorous precursors in grapes, can form the axis for the varietal sensorial expression of the wine bouquet).

For the analysis of terpene glycosides (non odorous potential precursors of terpenes), the fractionated extraction (obtained through elution in diffrent steps at different percentage of methanol) with C18 SPE needed in order to allow a reduction of interferences and then to facilitate the detection of these compounds for neutral (non-

aromatic) grapes. In order to obtain a structural evidence of the presence and of the identity for terpene glycosides, information was obtained by means of a combined use of available spectrometric techniques (GC/MS, LC/MS).

As to the LC/ESI/MS analysis on native aroma precursors (terpene glycosides) the best results were obtained in negative ion mode rather than in positive ion mode.

The use of discriminant analysis resulted instrumental in providing the identification of some possible odorous markers for typical wines.

Further studies can be carried out to take advantage of the utmost potentialities of varietal aromatic characteristics of typical wines; correlations between environmental characteristics (soil, climate, etc.) and varietal aromatic potentialities of grapes could be studied; exogenous enzymes, could be considered to liberate better varietal aroma compounds in wines.

Aging and storage give wine the time for the slow transformation of the free and bound monoterpenes.

Storage conditions (time, temperature, light) seemed critical to preserve varietal aroma compounds of typical wines.

The analysis of mannoproteins extracted from different wine samples showed large quantitative differences depending on different release potentialities of the yeasts used on the different winemaking process.

A large variability in the structural characteristics (strictly related to the enological functions of mannoprotins) was observed: heterogeneity in the peptide sequences and in glycosyl residues.

It could be interesting to correlate structural characteristics of parietal mannoproteins present in wines and their potential release during wine making process with particular selected strains of yeasts.

The aromatic characterization of Mozzarella di Bufala Campana cheeses from different producers of region Campania showed large differences in the composition of short chain FFA and other odorous molecules.

The production of these compounds primarily depend on the enzyme content of the strains. For example, some LAB strains produce aldehydes and alcohols as a result of an α-keto acid decarboxylase activity, while some other strains mainly produce carboxylic acids and hydroxy acids.The production of these metabolites is also affected by environmental factors such as pH and NaCl concentration, as these factors affect the enzyme activities. The aroma profile of this typical cheese appeared not yet "standardized" because of differences in natural whey starters used and in the making process carried out according to D.O.P. production disciplinary.

Differences in terpene composition were also observed in the aroma profile of Mozzarella di Bufala cheeses; these compounds derive from specific animal feeding.

Selected starters and adeguate feeding for animals can improve the sensorial quality of Mozzarella di Bufala Campana typical cheese.

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Odorous molecules	BN Falanghina variety	CF Falanghina variety	Coda di Volpe variety
limonene	X	X	X
cis-linalool oxide	X	X	
geraniol	X	X	
4-carene	X	X	
myrcene	X	X	
linalool	X	X	X
α-terpineol	X	X	X
menthol	X	x	
bornyl acetate	X	x	X
β-damascenone	X	X	
α-ionone	X	X	

Table 1 Composition of varietal odorous compounds of Falanghina (BN. Benevento; CF. Campi Flegrei) and Coda di Volpe varieties (X→ present; x→ present in traces only).

GLYCOSIDES	Trebbiano Toscano grape	Falanghina C.F. grape	Falanghina BN grape
glucopyranoside of linalool		x	X
glucoside of linalool oxide (a)	X	X	X
glucoside of linalool oxide (b)	X	X	X
rhamnopyranosyl glucoside of linalool	x	X	x
rhamnopyranosyl glucoside of geraniol	X	X	X
arabinofuranosyl glucoside of geraniol	X	X	X
arabinofuranosyl glucoside of linalool	x	X	X
X present			
x traces			

Table 2 Terpene glycosides identified in some different grapes.

	Greco grape	Fiano grape	Asprinio grape
α-pinene	-	+	-
camphene	-	+	-
β-pinene	-	+	-
myrcene	+	+	-
limonene	+	+	tracce
3-carene	+	+	+
4-carene	+	+	-
linalool	+	+	tracce
menthol	+	-	-
α-terpineol	-	+	-
geraniol	-	+	-

Table 3 Preliminary results obtained for the terpene composition of different autochthonous grapes of region Campania.

Aroma compounds	Odorous descriptor	Threshold (mg/l)
esters		
ethyl butanoate	Strawberry, apple, banana	0.4
ethyl hexanoate	Fruity, green apple, banana, brandy, wine-like	0.08
ethyl octanoate	Ripe fruits, pear, sweety	0.24
ethyl decanoate	Sweety, fruity, dry fruits	1.10
ethyl dodecanoate	Fruity	
butyl octanoate	Fruity	
isopenthyl hexanoate	Fruity	
hexyl acetate	Apple, cherry, pear, floral	0.67
1-butanol, 2-methyl acetate	Fruity	
1-butanol, 3-methyl acetate	Banana, fruity, sweet	0.16
ethyl succinate	Cheese, earthy, spicy	
2-phenyl ethyl acetate	Fruity, floral, rose	0.25
methyl decanoate	Fruity	
3-methyl, butyl octanoate	Fruity	
butyl butanoate	Fruity	
2-hydroxy ethyl propanoate	Fruity	
ethyl nonanoate	Fruity	
acids		
hexanoic acid	Rancid, grass, fruity	6.70
octanoic acid	Fatty acid, dry, dairy	2.20
decanoic acid	Fatty acid, dry, woody	1.40
dodecanoic acid		
alcohols		
2,3-butandiol		
3-methyl, 1-butanol		
phenyl ethyl alcohol	Flowery, rose, honey	10.0
1-hexanol	Herbaceous	4.80
benzyl alcohol	Flowery-sweet	
aldehydes		
benzaldheyde	Almond, fragant	2
4-methyl, benzaldehyde		
2-methyl, benzaldehyde		
terpenes		
α-terpineol	Lilac, floral, sweet	1
cis-linalyl oxide		
linalool	Citrus, floral, sweet, grape-like	0.015
geraniol	Rose	
4-carene	Citrus	
myrcene	Spice	
limonene	Lemon	
norisoprenoids		
β-damascenone	Balsamic, rose, violet	
α-ionone	Floral	
terpene derivatives		
menthol	Spice	
bornyl acetate	Spice	

Table 4 Odorous molecules detected for Falanghina wines.

Variables	Fuction	
	1	2
A	-3,318	,223
B	1,332	-1,093
C	-,002	-,017
D	,808	-,646
E	,226	,298
G	2,195	1,378
H	1,801	,818

Table 5 Standardized coefficients of discriminant functions.

number of cases	effective group	Betst forecasted group					Second best forecasted group			Discriminant score	
		forecasted group	(D>d/G=g) p	df	P(G=g/D=d)	Mahalanobis Distance (squared distance from barycentre	group	P(G=g/D=d)	Mahalanobis Distance (squared distance from barycentre)	function 1	function 2
1	1	1	.335	2	.000	2.190	2	.000	21.280	2.240	-1.862
2	1	1	.441	2	.999	1.639	3	.001	15.708	-.437	-2.086
3	1	1	.802	2	1.000	.442	3	.000	28.759	.674	-2.945
4	2	2	.849	2	1.000	.327	1	.000	42.745	6.129	1.526
5	2	2	.317	2	1.000	2.296	1	.000	20.626	4.415	.486
6	2	2	.542	2	1.000	1.225	1	.000	48.888	6.928	1.116
7	3	3	.849	2	1.000	.328	1	.000	31.330	-3.597	1.132
8	3	3	.610	2	1.000	.989	1	.000	18.791	-3.033	-.323
9	3	3	.506	2	1.000	1.363	1	.000	38.925	-4.191	1.411
10	3	3	.563	2	1.000	1.148	1	.000	24.225	-3.704	-.375
11	3	3	.165	2	1.000	3.600	1	.000	38.409	-3.085	2.510
12	3	3	.294	2	.995	2.451	1	.005	12.939	-2.339	-.588

Table 6 Synthetic information about the discriminant analysis.

Parietal mannoprotein of Saccharomyces cerevisiae	M.W. (Da)
Q07987	11382
P38155	10560
P47179	114622
P40552	24161
P28319	20274

Table 7 Mannoproteins identified in wine samples analysed.

Odorous molecules	A	B	C	D	E
terpene compounds					
bornyl acetate	X	X	X	X	X
α-pinene	X			X	X
camphene	X				
β-pinene	X			X	
3-carene	X		X	X	X
4-carene	X		X	X	
linalolool	X			X	
α-terpineol	X				
geraniol	X				
limonene	X	X	X	X	
β–myrcene				X	
borneol					X
esters					
ethyl butanoate	X	X			
isoamyl acetate	X				X
ethyl hexanoate	X	X			
ethyl octanoate	X	X		X	X
ethyl decanoate	X	X			
acids					
butanoic acid	X	X	X	X	X
hexanoic acid	X	X	X	X	X
octanoic acid	X	X	X	X	X
decanoic acid	X	X	X	X	X
dodecanoic acid	X	X	X	X	X
aldehydes					
hexanal	X		X	X	X
nonanal	X	X	X	X	
heptanal	X		X		
decanal	X	X	X	X	X
octanal	X			X	X
benzaldehyde	X			X	X
4-methyl benzaldehyde					X
2-undecenal					X
ketones					
5-methyl, 2-hexanone	X		X		
2-heptanone	X	X		X	
2-nonanone	X	X		X	X
2-dodecanone		X			X

Table 8 Comparison of the composition in odorous molecules for Mozzarella samples from different producers of region Campania.

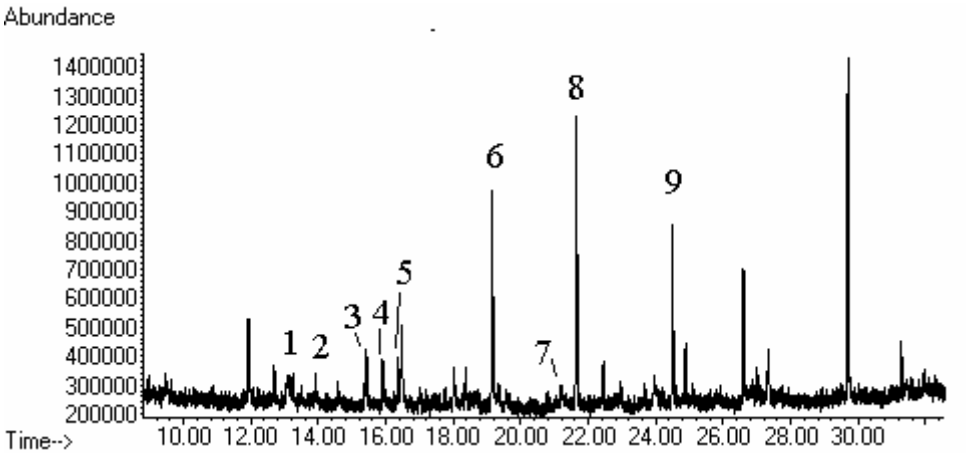

Fig. 1 TIC chromatogram obtained by means of static headspace-GC/MS analysis in Falanghina grapes: 1. myrcene; 2. limonene; 3. linalyl oxide; 4. 4-carene; 5. linalool; 6. α-terpineol; 7. geraniol; 8. α-ionone; 9. β-damascenone.

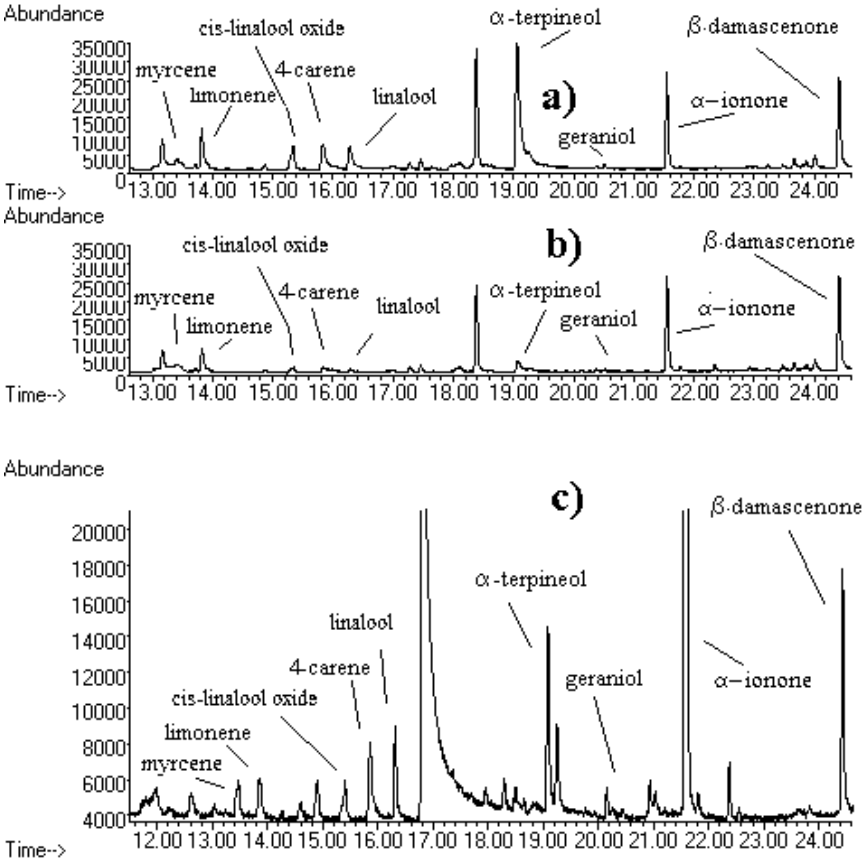

Fig. 2 Terpenes and norisoprenoids identified by means of static headspace-GC/MS analysis in Falanghina grapes of Beneventano area a), Campi Flegrei area b), and identified in Falanghina wine sample c) by means of SPME-GC/MS analysis (SIM method; m/z 93, 121, 136).

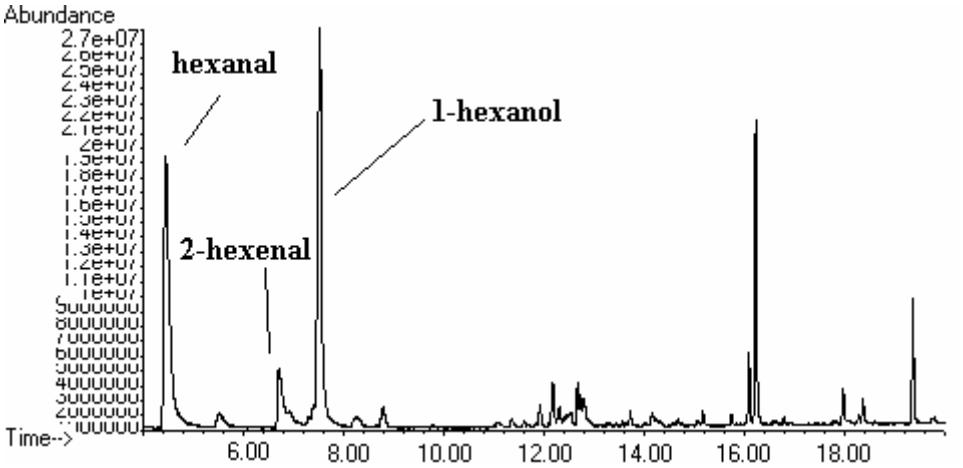

Fig. 3 TIC chromatogram obtained by means of SPME-GC/MS analysis for a sample of Falanghina grape.

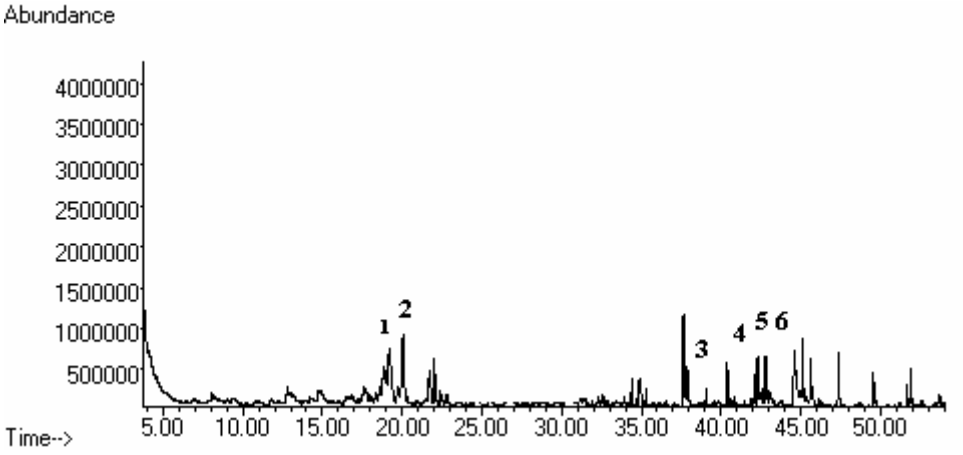

Fig. 4 TIC chromatogram obtained by means of GC/MS analysis of TMS-derivatives of terpene glycosides for an extract of Falanghina grape (1. linalyl glucoside; 2. geranyl glucoside; 3. linalyl arabinosylglucoside; 4. geranyl arabinosylglucoside; 5. linalyl rhamnosylglucoside; 6. geranyl rhamnosylglucoside).

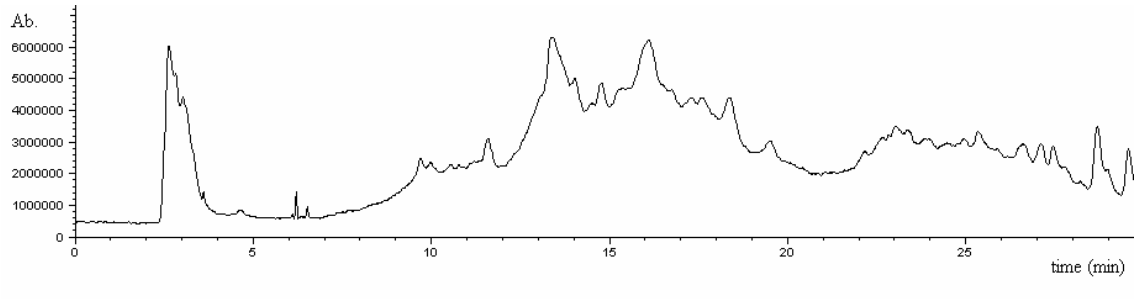

Fig. 5 TIC chromatogram obtained in negative ion mode by means of LC/ESI/MS analysis for an extract of Falanghina grape.

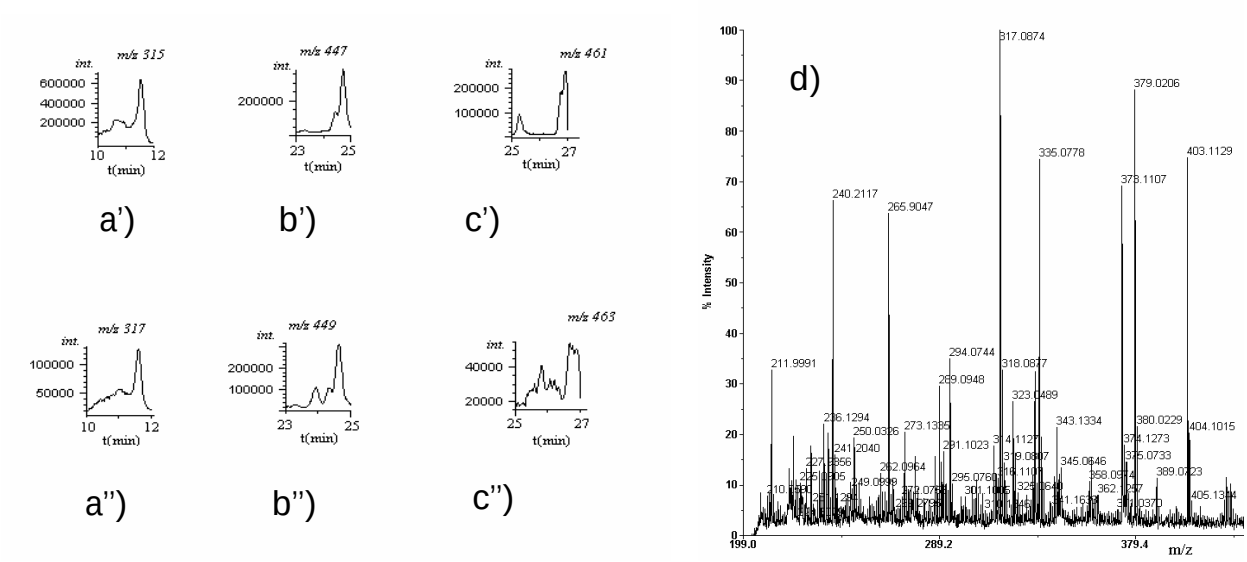

Fig. 6 Signals obtained by means of LC/ESI/MS analysis with an extract of grape in negative ion mode (a', b', c') and in positive ion mode (a'', b'', c'') (a. linalyl glucoside; b. linalyl arabinosylglucoside; c. linalyl rhamnosylglucoside), and through MALDI/TOF/MS analysis (d).

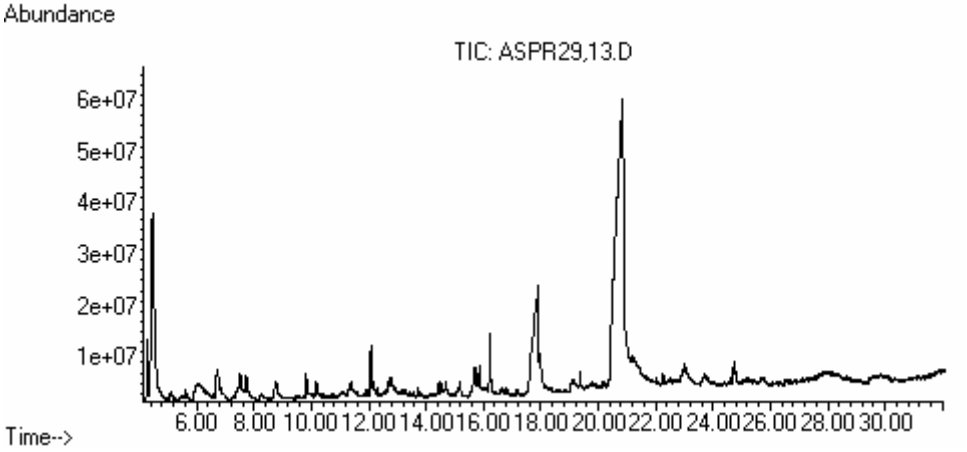

Fig. 7 TIC chromatogram obtained by means of SPME-GC/MS analysis for a sample of Asprinio grape.

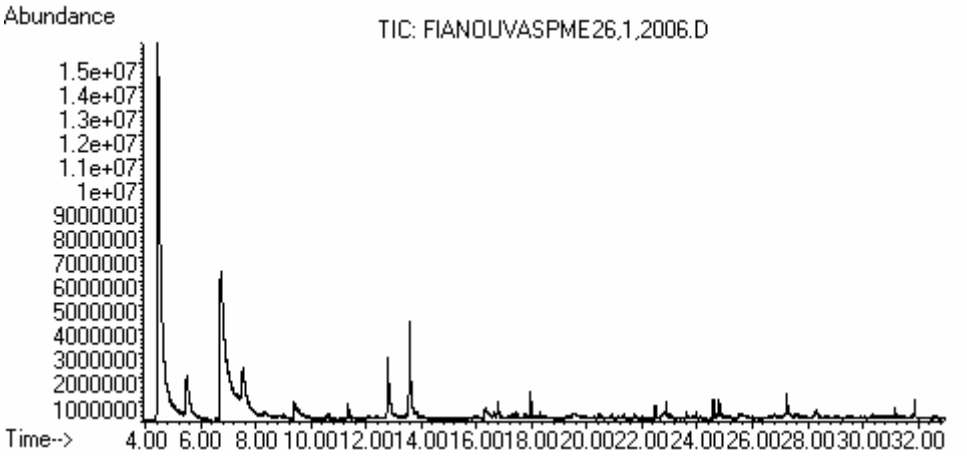

Fig. 8 TIC chromatogram obtained by means of SPME-GC/MS analysis for a sample of Fiano grape.

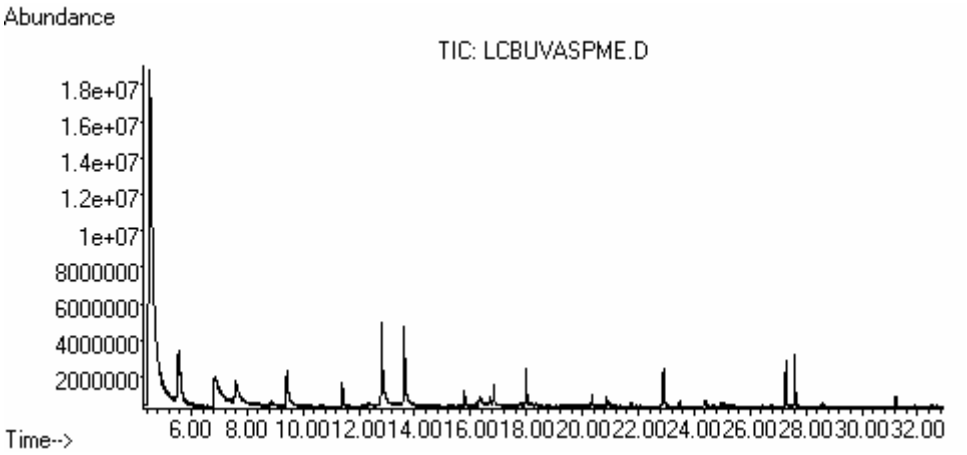

Fig. 9 TIC chromatogram obtained by means of SPME-GC/MS analysis for a sample of Coda di Volpe grape.

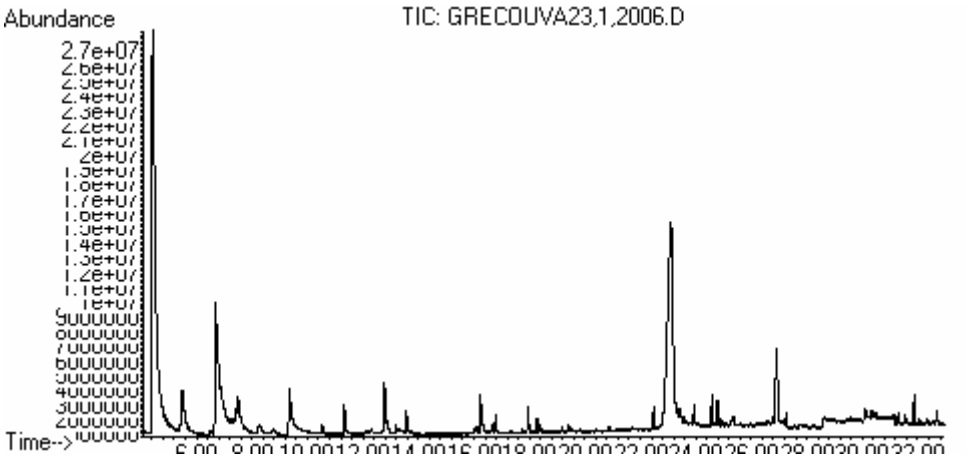

Fig. 10 TIC chromatogram obtained by means of SPME-GC/MS analysis for a sample of Greco grape.

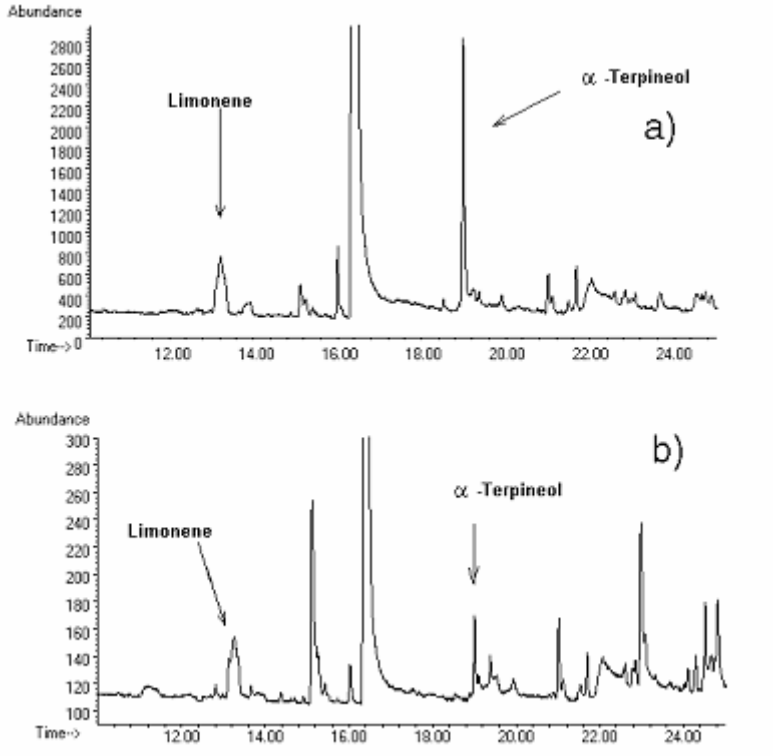

Fig. 11 SIM (m/z 93, 121, 136) chromatograms obtained by means of gas chromatography mass spectrometry analysis, for Falanghina wines from Beneventano (a) and from Campi Flegrei (b).

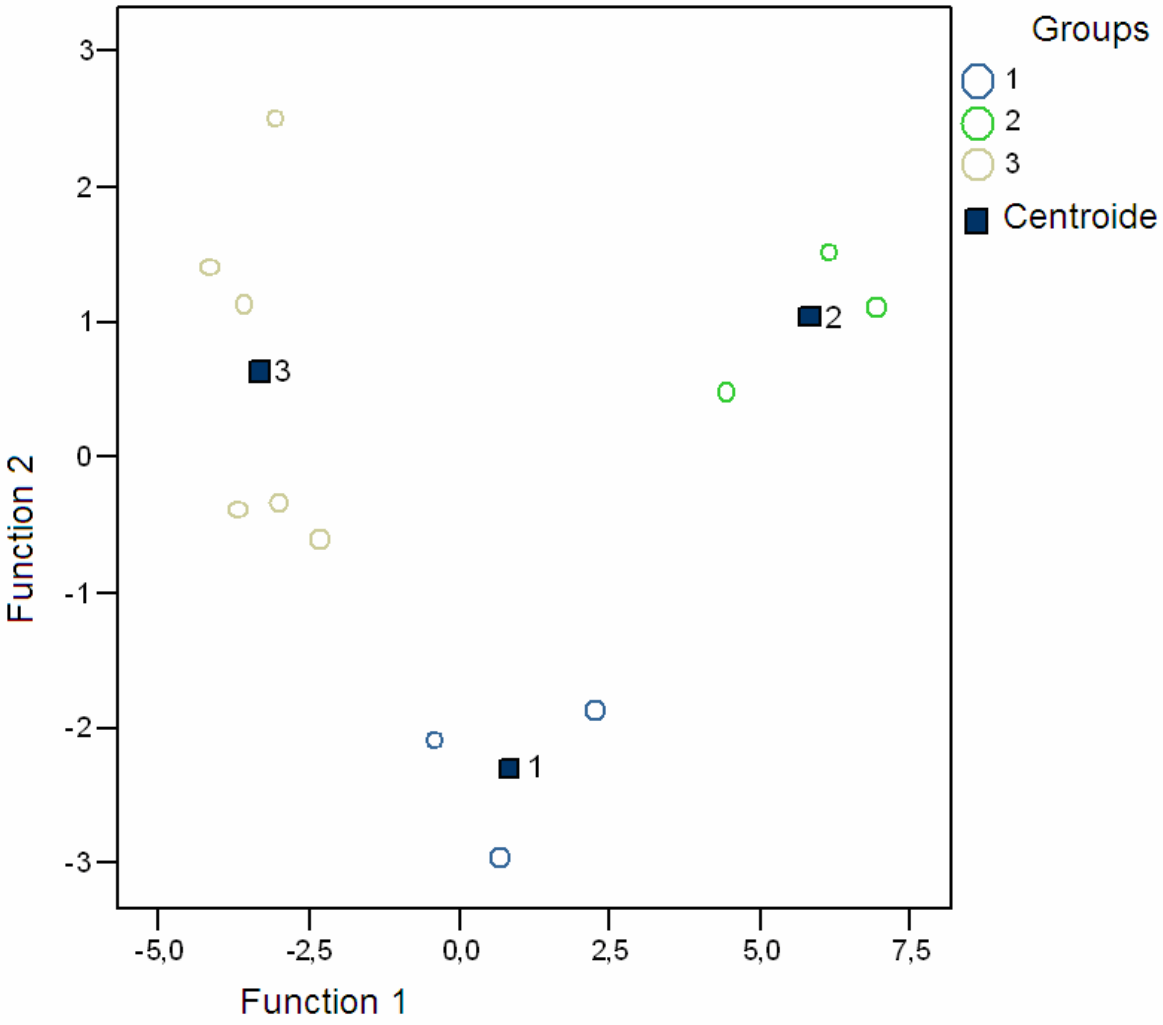

Fig. 12 Discriminant analysis carried out with the quantitative data of common aromatic molecules in samples of Falanghina wines (group 3) and other two typical wines (Lacryma Christi and Greco di Tufo)(group 1, group 2).

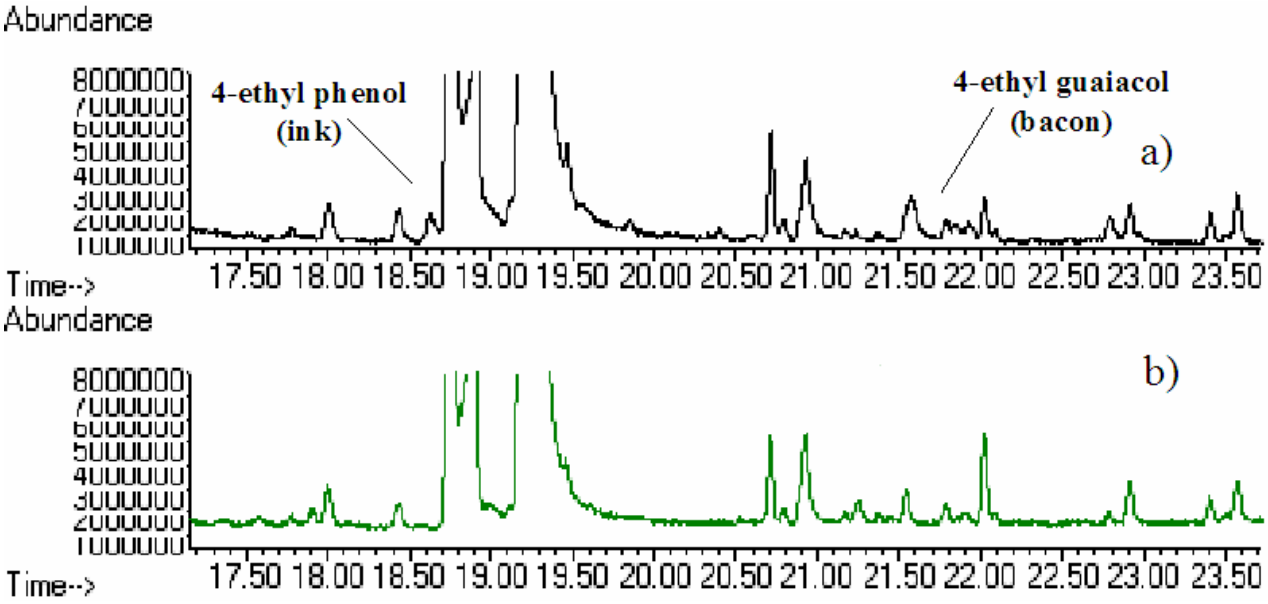

Fig. 13 TIC chromatograms obtained for fiano wine aged in barrique a) and in steel b).

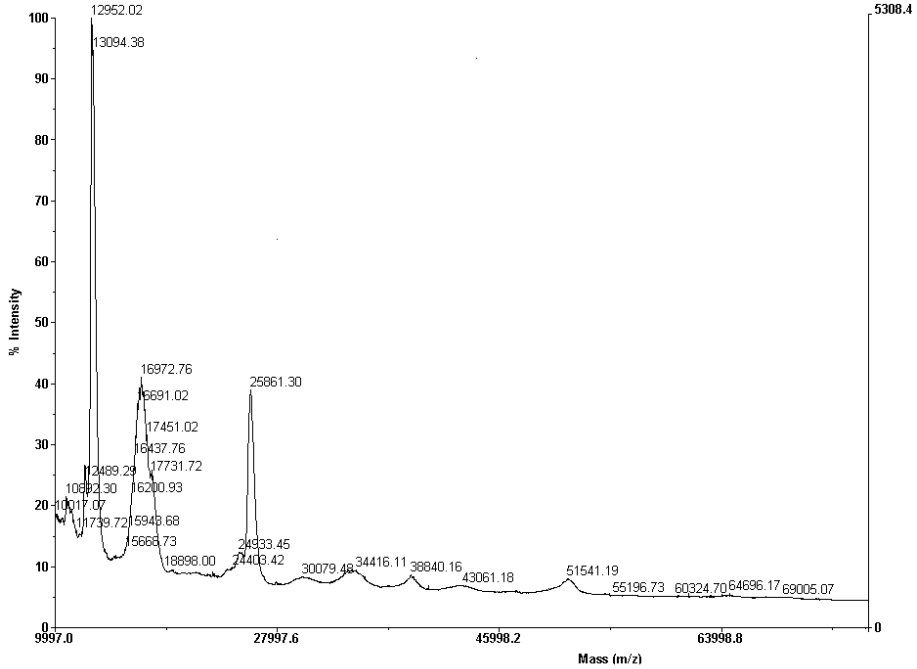

Fig. 14 MALDI TOF spectrum of mannoproteins from a sample of Falanghina wine.

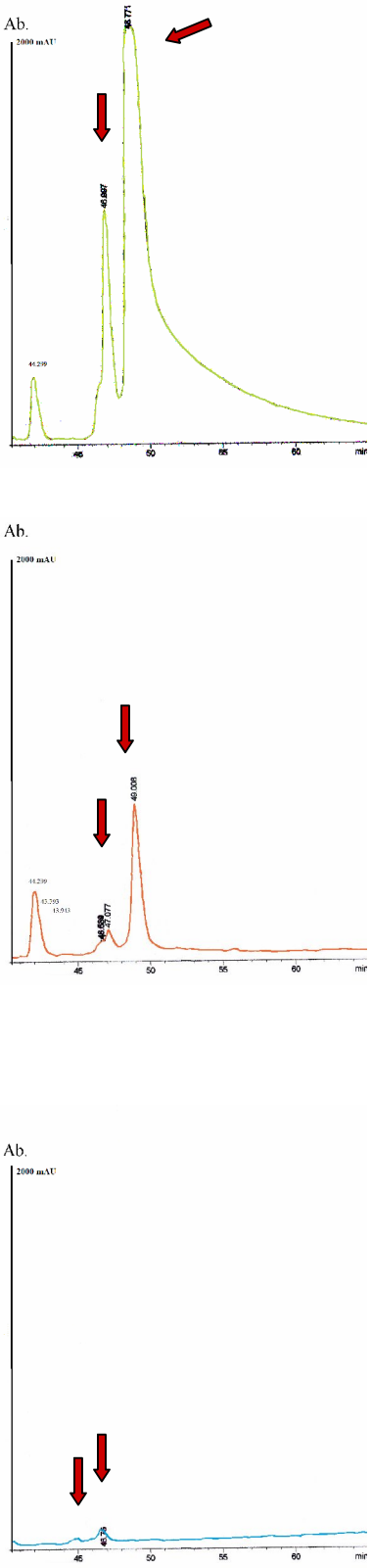

Fig. 15 HPLC chromatograms of mannoprotein extracts from some wines (Fiano, Falanghina, Asprinio).

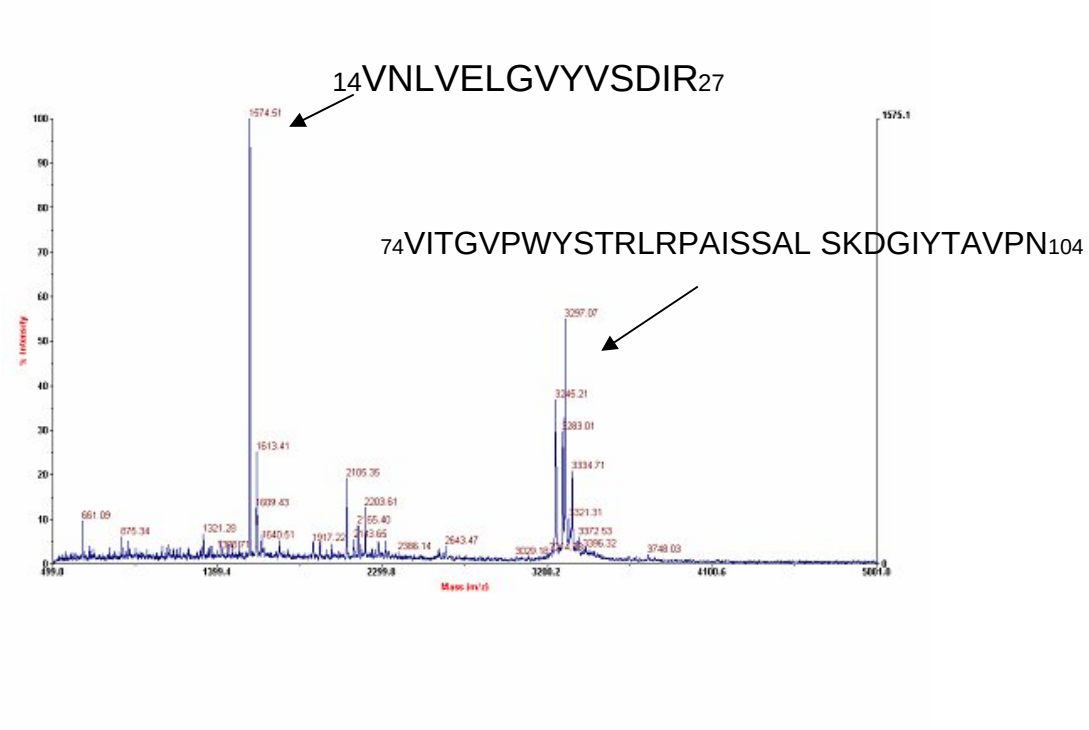

Fig. 16 MALDI TOF MS spectrum obtained for a tryptic digest of mannoproteins extracted from different wine samples.

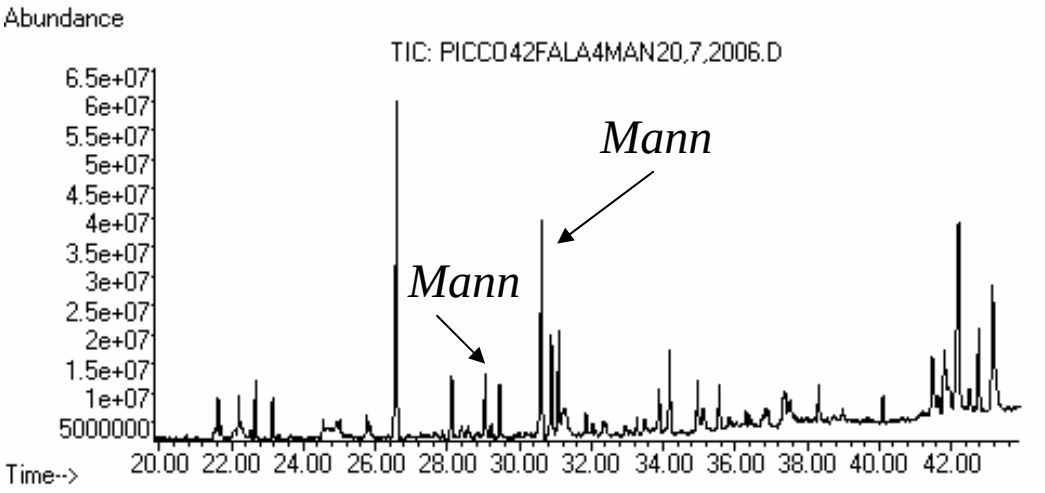

Fig. 17 GC/MS analysis of TMS-derivatives of glycosyl-residues of mannoproteins.

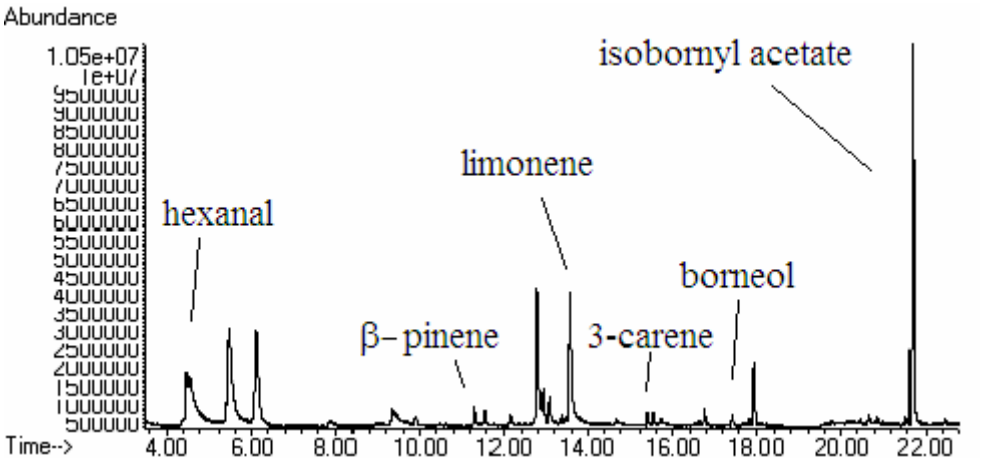

Fig. 18 TIC chromatogram obtained by means of HS-SPME-GC/MS analysis for a Mozzarella di Bufala Campana sample.

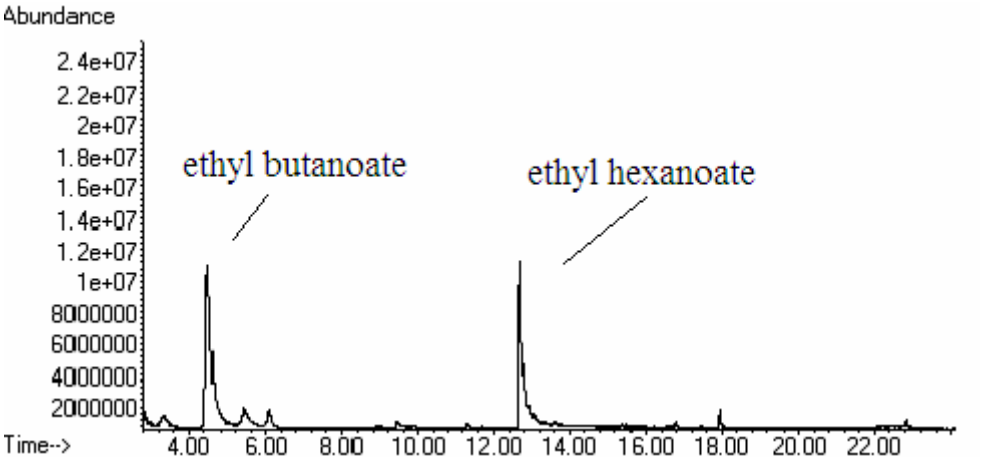

Fig. 19 TIC chromatogram obtained by means of HS-SPME-GC/MS analysis for a Mozzarella di Bufala Campana cheese sample.